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<b>(54) Title:</b> METHOD FOR PRODUCING HUMAN ANTIBODIES IN A NON-HUMAN ANIMAL, AND ANIMALS THEREFOR  <b>(57) Abstract</b>  The present invention relates to methods for producing human antibodies in a non-human immunodeficient animal such as an immunodeficient mouse. In addition, the invention describes methods for producing a non-human animal having a recon- stituted human immune system useful for producing human antibodies.		

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METHOD FOR PRODUCING HUMAN ANTIBODIES  
IN A NON-HUMAN ANIMAL, AND ANIMALS THEREFOR

Description

5

Technical Field

The present invention relates to a method for producing human antibodies in a non-human animal by reconstituting a human immune system in the animal.  
10 The invention also relates to a non-human animal having a reconstituted human immune system.

Background

in vivo human studies are limited by both  
15 practical and ethical considerations. Therefore, attempts have been made to recreate a human immune system in small laboratory animals. These involved the use of chimeric models of human bone marrow transfer into immunodeficient mice due to immune-impairing  
20 mutations (beige/nude/xid mice; Kamel-Reid et al, Science, 242:1706-1709, 1988), or lethally-irradiated BALB/c mice (Lubin et al, Science, 252:427-431, 1991). Homozygous C.B-17-scid/scid (SCID) mice (Bosma et al, Nature, 301:527-530, 1983), which lack DNA repair  
25 (Fulop et al, Nature, 347:479-482, 1990) and recombinase enzyme system functions (Schuler et al, Cell, 46:963-972, 1986; Malynn et al, Cell, 54:453-460, 1988; Hendrickson et al, Genes and Development, 2:817-829, 1988; Lieber et al, Cell,  
30 55:7-16, 1988; and Blackwell et al, EMBO J., 8(3):735-742, 1989) , have also been used. These mice are deficient in B and T cell(s) (functions) (Bosma et al, Nature, 301:527-530, 1983; and Custer et al, Am. J. Pathol., 120(3):464-477, 1985) , and have been  
35 proven to sustain the differentiation of mature human

B and T cells from human fetal lymphoid xenografts (McCune et al, Science, 241:1632-1639, 1988).

5 C.B-17-scid/scid mice lack B and T cells and their associated functions and, therefore, suffer from a severe combined immunodeficiency (SCID) disease . Bosma et al, Nature, 301:527-530 (1983). Such mice have been successfully populated with human fetal lymphoid cells (SCID-hu) (McCune et al, Science, 241:1632-1639, 1988), and human peripheral blood  
10 leukocytes (hu-PBL-SCID) (Mosier et al, Nature, 335:256-259, 1988; Mosier et al, Nature, 338:211, 1989; and Mosier et al, International Application No. PCT/US89/02591) to create an in vivo human laboratory model for the study of retroviral infection-associated  
15 disease (Mosier et al, Science, 251:791-794, 1991), autoimmune diseases (Krams et al, J. Exp. Med., 170:1919-1930, 1989; Tighe et al, Eur. J. Immunol., 20:1843-1848, 1990; and Duchosal et al, J. Exp. Med., 172:985-988, 1990), and human B cell lymphomagenesis  
20 (Cannon et al, J. Clin. Invest., 85:1333-1337, 1990; Okano et al, Am. J. Pathol., 137:517-522, 1990; Rowe et al, J. Exp. Med., 173:147-158, 1991).

However, only a limited number of terminally differentiated human Ig-producing B cells are present  
25 in the original hu-PBL-SCID mouse model (Saxon et al, J. Clin. Invest., 87:658-665, 1991), and the human PBL xenograft usually does not create a graft-versus-host (GvH) disease (Mosier et al, Nature, 335:256-259, 1988). A controversial and potentially limiting  
30 aspect of these models is the degree of functionality of the xenotransplanted human graft (McCune et al, Science, 241:1632-1639, 1988; Mosier et al, Nature, 335:256-259, 1988; Mosier et al, Nature, 338:211, 1989; Krams et al, J. Exp. Med., 170:1919-1930, 1989;  
35 Tighe et al, Eur. J. Immunol., 20:1843-1848, 1990; and

Bankert et al, Current Topics in Microbiology and Immunology, 152:201-210, 1989).

Brief Summary of the Invention

5           A method has now been discovered that provides for more complete population of a SCID mouse recipient such that the reconstituted mouse exhibits human IgG serum levels of typically 2 to 5 grams per liter in the absence of human antibodies immunoreactive with  
10   Epstein-Barr virus antigens. The reconstituted hu-PBL-SCID mouse produced by the present invention can respond immunologically and produce substantial specific human IgG upon immunization with a preselected antigen.

15           Thus, in one embodiment, the invention contemplates a method for producing human antibodies that immunoreact with a preselected antigen in a severe combined immunodeficiency (SCID) mouse comprising the steps of isolating a suspension of  
20   viable human peripheral blood lymphocytes (PBL) from a human donor, contacting the viable PBL in a medium in which lymphocytes are viable with the preselected antigen in an amount sufficient for the antigen to induce an immune response in the PBL,  
25   intraperitoneally introducing the PBL into a mouse having severe combined immunodeficiency, thereby forming a hu-PBL-SCID mouse having a reconstituted human immune system, and immunizing the said hu-PBL-SCID mouse with the antigen in an amount sufficient to  
30   induce an immune response wherein human antibodies immunoreactive with the antigen are formed in the hu-PBL-SCID mouse.

          The invention also contemplates a reconstituted hu-PBL-SCID mouse containing human lymphocytes, such  
35   that the hu-PBL-SCID mouse has a plasma concentration

of 2 to 5 grams per liter of human immunoglobulin and plasma that is seronegative for Epstein-Barr virus.

5 A further embodiment contemplates a method for producing a hu-PBL-SCID mouse having a reconstituted human immune system that is defined by a plasma concentration of human immunoglobulin of 2 to 5 grams per liter comprising the steps of lymphopheresing blood from a human donor to produce lymphopheresed cells containing lymphocytes, red blood cells and plasma, centrifuging the lymphopheresed cells through 10 a medium in which lymphocytes are viable at a gravity force and for a time sufficient to pellet the lymphocytes but not the red blood cells and plasma present in the lymphopheresed cells and form pelleted lymphocytes and a RBC/plasma-containing supernatant, 15 separating the pelleted lymphocytes from the supernatant to form isolated PBL, resuspending the isolated PBL in a medium in which lymphocytes are viable to form the suspension of isolated viable PBL, 20 and introducing the isolated viable PBL into a mouse having severe combined immunodeficiency, thereby forming a hu-PBL-SCID mouse having a reconstituted human immune system.

In a related embodiment, the invention 25 contemplates a method for producing a hu-PBL-SCID mouse as above comprising the steps of centrifuging heparinized whole blood from the donor through a high density ficoll medium of density 1.119 gram per milliliter to form upper, lower and inter layers, 30 recovering the inter layer containing lymphocytes, red blood cells and plasma, diluting the inter layer with an equal volume of medium in which lymphocytes are viable, centrifuging the diluted inter layer as above to pellet the lymphocytes but not the red blood cells or plasma and form pelleted lymphocytes and a 35

RBC/plasma-containing supernatant, separating the pelleted lymphocytes from the supernatant to form isolated lymphocytes, resuspending the isolated lymphocytes in medium in which lymphocytes are viable to form the suspension of isolated viable PBL, and introducing the isolated viable PBL into a mouse having severe combined immunodeficiency, thereby forming a hu-PBL-SCID mouse having a reconstituted human immune system.

#### Brief Description of the Drawings

Figure 1 illustrates the time course evolution of mean human IgG serum levels in 66 SCID mice populated with  $15 \times 10^6$  PBL from an EBV donor [---], 9 mice irradiated to 250 rads 12 hours before receiving  $15 \times 10^6$  PBL from the same donation [\_\_\_], and 11 mice receiving  $15 \times 10^6$  PBL from the same donation deep frozen for 3 days [...].

Figures 2A through 2E illustrate the immunization with TT antigen. Figure 2A shows a comparison of TT serum titer for a donor (#4) not boosted for 20 years (open circles) with that ( $\pm$ SEM) obtained by boosting the donor's PBL in 6 hu-PBL-SCID mice (closed circles). These 6 hu-PBL-SCID mice had, 29 days post cell transfer,  $2.86 \pm 1.19$ , and  $6.36 \pm 3.34$  IU of mean absolute-, and normalized-IgG anti-TT/ml, respectively. The mean values for 3 similarly populated mice but not immunized is represented (closed triangles). This ELISA did not show any reactivity when sera from non-populated SCID, BALB/c, or C57BL/6 mice were applied.

Figures 2B and 2D show the IgG anti-TT serum response in hu-PBL-SCID mice non-immunized with TT. Figures 2C and 2E show the anti-TT serum response in hu-PBL-SCID mice immunized with TT. Mice were populated

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with PBL from either donor #5 (B,C), immune to TT with an IgG anti-TT level of 0.88 IU/ml, not boosted with TT for at least 4 years, or from donor #6 (D,E), immune to TT, and boosted with TT (Wyeth) 6 days prior to PBL transfer. IgG anti-TT serum levels of donor #6 at day 0, and day 37 were 0.65, and 7.60 IU/ml, respectively. Donors #4, #5, and #6 have IgG serum levels of 8.49, 8.12, and 9.82 g/l, enabling comparisons between groups of hu-PBL-SCID mice normalized anti-TT levels. Each dot corresponds to an individual bleed. Whenever appropriate, median values are indicated by horizontal bars.

#### Detailed Description of the Invention

The present invention is founded on two discoveries. First, it is shown that a method of introducing human lymphocytes into an immunodeficient non-human animal according to the present invention produces a non-human animal having a reconstituted human immune system which is capable of producing higher plasma levels of human immunoglobulin than previously reported in the absence of human antibodies immunoreactive with EBV antigens. Second, it is shown that a method of immunizing the reconstituted human immune system according to the present invention induces a specific immune response to the preselected immunizing antigen.

#### A. A Non-Human Immunodeficient Animal Having a Reconstituted Human Immune System

The present invention contemplates a non-human animal having a reconstituted human immune system. The reconstituted human immune system is comprised of a xenogenic transplant of human peripheral blood lymphocytes (PBL) and thus the reconstituted animal is



referred to herein as a hu-PBL animal. In preferred embodiments, the non-human animal is a mammal such as a mouse, hamster, rat, dog, cat, goat, monkey or the like mammal, and is more preferably a mouse.

5           The recipient non-human mammal must be an immunodeficient animal in order that the xenogenic transplant succeed. Immunodeficiency in a mammal can be provided by a variety of means such as by genetic deficiency or by irradiation of the recipient animal.  
10 Immune-impairing genetic deficiencies are known for a variety of the above recited species, for example the beige/nude/xid mouse (Kamel-Reid et al, Science, 242:1706-1709, 1988) or the severe combined immunodeficiency (SCID) mouse. Particularly preferred  
15 is the homozygous C.B-17-SCID/SCID (SCID) mouse (Bosma et al, Nature, 301:527-530, 1983). A preferred irradiation-induced immunodeficiency is the lethally-irradiated BALB/c mouse described by Lubin et al, (Science, 252:427-431, 1991). SCID mice are available  
20 from commercial vendors and the research community in their mouse breeding facility.

A human immune system-reconstituted non-human animal that is described as exemplary herein that is particularly preferred is the SCID mouse, and is  
25 referred to herein as a hu-PBL-SCID mouse.

A hu-PBL-SCID mouse of this invention is defined by a plasma concentration of human gamma globulin (HGG) of 1 to 8 grams per liter (g/l), preferably 2 to 5 g/l. Assays to determine plasma levels of HGG are  
30 well known in the art. An exemplary assay for HGG is described by Duchosal et al, in J. Exp. Med., 172:985-988 (1990).

In preferred embodiments, a hu-PBL-SCID mouse of this invention is seronegative for human antibodies  
35 immunoreactive with antigens of the Epstein-Barr Virus

(EBV). EBV serology can be determined by any of the well established blood bank protocols that are commercially available. Preferably, the donor does not contain latent EBV infection, as can be determined by nucleic acid hybridization. It is well established by earlier work by Mosier and others using PBL from EBV seropositive (EBV<sup>+</sup>) or EBV seronegative (EBV<sup>-</sup>) donors that human B cell lymphomas can develop in the reconstituted mouse when the donor is EBV<sup>+</sup>, particularly when the SCID mouse has received the higher published numbers of PBL in the transplant.

In addition, by previously published methods for preparing a human immune system in a SCID mouse, an EBV<sup>+</sup> donor was required in order that the resulting xenogenic hu-PBL-SCID mouse contain plasma human immunoglobulin levels greater than about 0.1 to 1 gram per liter. See Mosier et al, International Application No. PCT/US89/02591.

Thus, the present invention provides a hu-PBL non-human animal containing plasma human gamma globulin levels of 1 to 7 g/l, and more typically levels of 2 or 3 to 5 g/l without the requirement for PBL from a EBV<sup>+</sup> donor.

This aspect of the invention provides unique and important advantages. First, as described herein, one benefit to the invention is the ability to obtain PBL from a human donor who is a patient in need of an antibody supplement, such as a passive immunization, but who is EBV<sup>-</sup>. According to the present invention, the EBV<sup>-</sup> donated PBL can be transplanted and the resulting hu-PBL-SCID mouse can be immunized according to the present invention to yield 1 to 8 g/l of plasma HGG which contains substantial levels of immunoglobulin specific for the immunizing antigen. Second, the preparation of therapeutic antibodies from

sources free from EBV is preferred for health safety reasons.

B. Methods for Producing a Hu-PBL Non-Human Animal

The invention contemplates a method for producing a hu-PBL non-human animal having a reconstituted human immune system. The method generally involves the steps of isolating PBL from a human donor according to the methods described below, and introducing the isolated PBL into an immunodeficient non-human animal to form a hu-PBL non-human animal.

By a reconstituted human immune system is meant that the animal contains human lymphocytes and exhibits a plasma concentration of human immunoglobulin from 1 to 8 g/l, and more typically from 2 to 5 g/l. In addition, a hu-PBL non-human animal containing a reconstituted human immune system has the capacity to respond immunologically to immunization as described herein and produce human antibodies immunospecific for the preselected immunizing antigen and described herein.

Methods for determining the presence of human lymphocytes are well known and involve the use of immunological reagents specific for human lymphocyte markers such as human leukocyte common antigen CD45, 2B11 and PD7/26, CD4, or other human specific lymphocyte markers.

The contemplated methods for preparing a hu-PBL non-human animal are exemplified herein by the detailed descriptions for preparing a hu-PBL-SCID mouse. However, the basic observations of PBL manipulations and immunization protocols described herein may be applied to the other non-human animals described.

### 1. Isolation of Human PBL

A hu-PBL-SCID mouse having a reconstituted human immune system is produced by first isolating PBL from a human donor, preferably an EBV seronegative donor.

5 The method of isolating the PBL can be accomplished by a variety of methods as discussed further below, but generally involves manipulations designed 1) to remove the red blood cells (RBC) and associated human plasma from the donor lymphocyte sample because

10 immunodeficient non-human recipients do not tolerate human plasma and RBC, 2) to minimize the trauma to the lymphocytes being isolated from the donor, and 3) to minimize the time the lymphocytes spend outside the body of either the donor or the recipient.

15 Two exemplary and preferred PBL isolation methods are described herein, which utilize either whole, . heparinized blood or lymphopheresed blood products, namely lymphopheresed cells.

The isolation of PBL from whole blood comprises  
20 the steps of first adding a clotting inhibitor such as heparin, citrate or the like as is well known in the hematological arts. Exemplary is the heparinized whole blood described in the examples. Thereafter, separation of the blood constituents is effected by  
25 differential centrifugation on a cell separating medium to form isolated lymphocytes. Typically this involves separation on a high density ficoll medium, as is well known.

A preferred ficoll medium for preparing human PBL  
30 has a density of 1.119 gram per milliliter (gm/ml) because the resulting inter layer contains macrophages, polymorphonuclear cells (PMN), lymphocytes, human plasma, and trace amounts of residual red blood cells. Previous ficoll mediums  
35 utilized for preparing PBL have a density of 1.077,

which separates the lymphocytes away from the macrophages and PMN.

Thus, the method for isolating PBL from whole blood comprises the steps of centrifuging heparinized whole blood from a human donor through a high density ficoll medium of density 1.119 gm/ml to form an upper, lower and inter layer. Centrifugation conditions for effecting the formation of the layers is dependent on the geometry of the medium, as is well known. A preferred ficoll medium of density 1.119 gm/ml is prepared using Histopaque-1119 according to the manufacturer's specifications (Sigma Chemical Co., St. Louis, MO).

Thereafter, the inter layer is collected (recovered) and diluted with a medium compatible with lymphocyte cell viability to allow washing of the collected lymphocytes.

A medium that is compatible with lymphocyte cell viability is a medium in which lymphocytes are viable, and can be subsequently shown to remain viable in culture. Preferably the medium is isotonic to minimize any adverse affects that in vitro culturing may have on the viability of the lymphocytes.

The degree of dilution is designed to facilitate a centrifugation step for removing residual red blood cells present in the inter layer. Volumes of diluent of about 0.5 to 2 times the inter layer volume are used, although equal volumes are preferred. A medium compatible with lymphocyte cell viability is any culture medium formulation designed for lymphocyte stabilization or culturing. Cell viability can be monitored by a variety of means including integrity of dye exclusion over time after exposure to the medium. Exemplary is Earle's medium described herein having 1 % bovine serum albumin (BSA).

The diluted inter layer is then subjected to centrifugation to selectively pellet the lymphocytes but not the RBC present in the inter layer, and thereby remove RBC and plasma. To that end, the  
5 diluted inter layer is subjected to centrifugation conditions comprising a gravity force and a time period sufficient to sediment (pellet) the lymphocytes without pelleting the RBC, forming pelleted  
10 lymphocytes and a supernatant containing the RBC and plasma. Exemplary centrifugation conditions sufficient for separation of RBC and plasma from lymphocytes in suspension are to subject the diluted inter layer to 1200 rotations per minute (RPM) on a  
15 Damon IEC EPR-6000 centrifuge at 4 degrees Centigrade (4C) for 10 minutes (min), using a swinging bucket rotor fitted with 50 ml buckets having a distance from center axis to tube bottom of about 12 inches. Other configurations could readily be substituted to preferentially pellet the lymphocytes.

20 The pelleted lymphocytes are then separated from the RBC/plasma-containing supernatant, typically by decanting the supernatant, to form isolated lymphocytes in pellet form.

25 Thereafter the isolated lymphocytes (in pellet form) are resuspended in a medium in which lymphocytes are viable, defined as before, and preferably of the same composition to form a suspension of isolated viable peripheral blood lymphocytes (PBL). The final  
30 volume of PBL in the suspension can vary widely, depending on the amount of PBL to be introduced into the recipient non-human animal, and the volume of cells that can be conveniently injected.

For the SCID mouse, typical injection volumes are from 0.2 to 2.0 ml, preferably about 1.0 ml. Typical  
35 cell concentrations in the isolated PBL suspension can

be from about  $4 \times 10^5$  to  $4 \times 10^7$  cells per ml, preferably are about  $10^6$  to  $250 \times 10^6$ , and more preferably are about  $50 \times 10^6$  cells per ml.

5       The isolation of viable PBL from lymphopheresed blood products comprises the steps of first obtaining fresh human lymphopheresed cells from a donor. Fresh cells are those which are collected from a human donor using an automated blood cell separator over a period of less than 6 hours, preferably less than 4 hours.  
10       Automated blood cell separators useful for conducting lymphopheresis include the Cobe 2997 unit (Cobe, Denver, CO) or the Fenwall CS 3000 (Fenwall, Deerpark, IL) used according to the manufacturer's instructions for effecting lymphopheresis to produce lymphopheresed  
15       cells, including PBL, human plasma and RBC.

Thereafter, the lymphopheresed cells are washed as before to remove RBC/plasma by centrifugation and resuspension as above to form a suspension of isolated viable PBL.

20               2.    Reconstituting a Human Immune System into a Recipient Animal

Following the above preparation of isolated viable human PBL, a hu-PBL non-human animal is produced by introducing the isolated PBL into a  
25       recipient immunodeficient non-human animal, thereby reconstituting the animal with a human immune system.

The recipient immunodeficient non-human animal used to exemplify the present invention is the SCID mouse described before.

30       Introduction of the previously isolated PBL is accomplished by injection of the suspension of isolated viable PBL intraperitoneally into a living, recipient SCID mouse, thereby forming a hu-PBL-SCID mouse having a reconstituted human immune system. The  
35       recipient SCID mouse is from about 4 to 12 weeks old,

and more preferably is 6 to 8 weeks old.

The amount of PBL to be injected can vary from 2 to  $50 \times 10^6$  cells, preferably from 10 to  $20 \times 10^6$  cells, and more preferably is about  $50 \times 10^6$  cells.

5        Insofar as the quality of the isolated viable PBL is important for the higher levels of effective xenogeneic transplant, measured as plasma levels of HGG, it is preferred if the injections occur after the minimum possible amount of time after removal of the  
10       PBL from the donor. Typical times range from about 1 to 6 hours, but are preferably within 2 to 4 hours.

As discussed previously, a preferred PBL for introduction is obtained from an EBV<sup>-</sup> seronegative donor.

15

C.    Methods for Producing Human Antibodies in an Immunodeficient Non-Human Animal

The present invention also contemplates methods for producing human antibodies in the reconstituted human immune system present in an immunodeficient non-  
20       human animal, such as in a hu-PBL-SCID mouse described herein. Particularly, the invention describes methods for inducing the human immune system to produce human antibodies that immunoreact with a preselected  
25       antigen, by exposing (contacting) the PBL of the reconstituted human immune system at particular times in the method to a preselected antigen, thereby "immunizing" the reconstituted human immune system. Thereafter, the "immunized" system produces human  
30       antibodies that immunoreact with the preselected antigen.

Thus the resulting human antibodies produced by the present methods have a preselected immunoreactivity similar to the reactivity obtained by  
35       conventional immunization of a mammal, with the



exception that the human donor is not exposed to the antigen in the course of the method of this invention.

To that end, the present methods for producing human antibodies in a hu-PBL immunodeficient animal of this invention comprise the steps of first isolating a suspension of viable human PBL as previously described. Preferably, the donor has a history of contact with the preselected antigen such that the population of isolated human PBL will contain memory B cells, and members of the population will have the potential, upon "immunization" (first and subsequent contacts with the preselected antigen outside the body of the donor) to respond immunologically in the form of a secondary immune response, expressing IgG class immunoglobulin immunospecific for the preselected antigen.

The second step in the method for producing human antibodies is to contact the isolated human PBL with a preselected antigen as to induce the secondary response, in a manner similar to in vivo immunization, with the exception being that the contact is not in the donor. Rather, the contact is before or at the time of xenogenic transplantation.

Thus in one embodiment that is exemplary herein, the isolated viable human PBL are cultured in vitro under in vitro immunizing conditions in the presence of the preselected antigen in an amount of antigen and for a time period sufficient for the antigen to induce an immune response in the PBL. in vitro immunizing conditions are human PBL culturing conditions, i.e., tissue culture at 37 degrees Centigrade in medium in which lymphocytes are viable, where the culture medium contains for at least a portion of the culturing time period an amount of the preselected antigen sufficient to induce an immune response. That amount of antigen

can vary widely so long as there is, upon introduction of the PBL into the recipient, a measurable increase in the ratio of specific human antibody over non-specific human antibody (HGG) in the plasma of the recipient over the four weeks post transplantation as demonstrated herein. The composition of the in vitro immunization conditions can include additional reagents designed to modulate the human B cell response as is well known, including growth hormones, stabilizing proteins, cytokines and the like. See, for example, the teachings in "Therapeutic Monoclonal Antibodies", Borrebaeck et al, Eds., Stockton Press, New York, NY, p.1-15, (1990).

Concentrations of the antigen and the human PBL to be induced ("immunized") in vitro can vary widely, but typically the lymphocyte concentration varies from  $0.4 \times 10^6$  to  $40 \times 10^6$  lymphocytes per ml of culture medium, preferably about  $1 \times 10^6$  to  $10 \times 10^6$  lymphocytes per ml, and more preferably about  $4 \times 10^6$  lymphocytes per ml. Similarly, the concentration of antigen can be from about 0.1 to 20 micrograms (ug) per ml, preferably about 1 to 5 ug/ml.

The time period for in vitro contacting (incubation) of the preselected antigen with the human PBL can vary also, although consideration of the integrity of the isolated PBL requires that the contacting be less than 6 hours, typically about 0.5 to 6 hours, and preferably about 4 hours. Exemplary in vitro culture medium, lymphocyte and antigen concentrations, and incubation time periods are described herein.

In a related embodiment, also demonstrated by the examples herein, the contacting can be accomplished by admixture of the isolated viable human PBL and the preselected antigen at the time of introduction of the

PBL into the recipient. That is, by either admixture of the antigen and the PBL immediately (within minutes) prior to injection, or by co-injection into the intraperitoneal cavity of the recipient, the first  
5 "immunizing" contact may be afforded. This latter embodiment is also referred to as "substantially simultaneously" carrying out the contacting and introducing steps of the method.

Following the first contacting of the human PBL with antigen in the course of the method, and the  
10 introduction of the human PBL into the recipient, one or more in vivo immunizations are conducted to boost the secondary immune response, manifest in the form of the production of human IgG in the blood of the  
15 recipient.

The timing of the immunizations, the amount of antigen and the carrier the antigen is delivered in, and the sites for immunization on the recipient can generally be varied as for conventional immunizations.  
20 However, typically a first immunization in complete Freund's adjuvant (CFA) followed by a second immunization in normal saline has produced the highest values of specific antibody production, as shown in the examples.

25 The amounts of preselected antigen utilized in the in vivo immunizations is typically in the range of 1 to 100 ug, preferably about 5 to 50 ug, and more preferably about 10 ug, although these amounts can vary depending on the molecular weight and  
30 immunogenicity of the antigen.

The time periods for immunizations in vivo can range from about days 0 to 21 for the first, and about days 7 to 28 for the second. Additional immunizations may also be conducted to further boost the response.  
35 Exemplary combinations of first and second

immunization are at days 2 and 21 or 22 or 23. Alternatively, a single in vivo immunization after introduction can be administered where the antigen is contacted with the PBL by co-injection into the peritoneal cavity.

Particularly preferred in vivo immunization protocols consistent with the present invention are described in the examples.

10           D.   Harvesting Human Antibodies from a Hu-PBL Immunodeficient Non-Human Animal

The human antibodies so produced in hu-PBL immunodeficient animal can then be harvested by a variety of means including 1) conventional collection of sera containing the human antibodies, 2) preparation of monoclonal antibodies by immortalizing the antibody producing human B cells of the hu-PBL immunodeficient animal, or 3) molecular cloning of the messenger RNA (mRNA) present in the human B cells of the hu-PBL immunodeficient animal and in turn preparing "repertoires" of molecular clones that each express a functional antibody molecule.

Conventional collection of sera is the simplest and quickest way to harvest the human antibodies produced by the methods of the present invention, and is shown in the examples.

Techniques for producing human monoclonal antibodies by immortalizing a human B cell through fusion with human or murine myelomas, or through transformation with EBV have been generally described in the art and can readily be applied to a human B cell obtained from a hu-PBL-SCID mouse of this invention. For discussions of the state of the art of producing human monoclonal antibodies from human B cells see Olsson et al, Meth. Enzymol., 92:3, (1983);

Buck et al, in "Monoclonal Antibodies and Functional Cell Lines: Progress and Applications", Kennet et al, Eds., Plenum Press, New York, NY, p. 275 (1984); and the teachings generally of "Monoclonal Antibodies: a  
5 Manual of Techniques", Zola,H.,CRC Press, Boca Raton, FL (1987), and of "Therapeutic Monoclonal Antibodies", Borrebaeck et al, Eds., Stockton Press, New York, NY (1990).

Techniques for producing monoclonal antibodies by  
10 the repertoire cloning of human B cell mRNA to form populations of nucleic acids containing immunoglobulin expression vectors have been described by a variety of groups. By use of these methods, also utilized herein and described in some detail in the examples, the  
15 isolation of a repertoire of nucleic acid sequences that encode a population of diverse immunoglobulin (antibody) molecule expressing clones derived through nucleic acid cloning from the human antibody producing B cells of the hu-PBL-SCID mouse was accomplished.

20 Typically, the nucleic acid sequences are produced by a polymerase chain reaction amplification of the immunoglobulin genes present in the repertoire of mRNA molecules present in the human B cells. The amplified immunoglobulin gene-specific nucleic acid  
25 sequences are manipulated into bacterial expression vectors for expressing the cloned immunoglobulin genes.

Populations of vectors are so constructed to form a repertoire of cloned immunoglobulin genes derived  
30 from a single mouse having human B cells responding to the single preselected immunizing antigen. The vectors are then inserted into a bacterial host, typically E.coli, and the bacteria are cultured under conditions for expressing the cloned immunoglobulin  
35 genes to produce functional heterodimeric antibody

molecules having assembled heavy and light chains.

The population of antibody molecule-expressing clones are screened for the production of antibody molecules that immunoreact with a preselected antigen.

5 In the examples, the antigen selected was tetanus toxin (TT). Those antibody-expressing clones producing preselected antigen-immunoreactive antibodies are selected for further growth, cultured in a clonal fashion and under conditions for  
10 expressing the selected antibody, and the resulting expressed antibody is collected from the culture medium of the clone, thereby yielding harvested human antibody.

For descriptions of the general state of the art  
15 of cloning and expressing antibodies from a repertoire of B cell derived antibody-encoding mRNA molecules, see the teachings of Huse et al, Science, 246:1275-1281 (1989); Sastry et al, Proc.Natl.Acad.Sci.USA, 86:5728-5732 (1989); Mullinax et al,  
20 Proc.Natl.Acad.Sci.USA, 87:8095-8099 (1990); and Persson et al, Proc.Natl.Acad.Sci.USA, 88:2432-2436 (1991).

#### Examples

25 The following examples are given for illustrative purposes only and do not in any way limit the scope of the invention.

##### 1. Isolation of Peripheral Blood Lymphocytes (PBL)

30 The aim of the transfer method was to minimize the number of manipulations required for PBL isolation, as well as to reduce the time between blood drawing, purification of PBL and PBL injection into SCID mice. Briefly, undiluted heparinized blood  
35 obtained from consenting donors was purified by

lymphopheresis after which the product was centrifuged for 15 minutes at 250 x g. Lymphopheresis was conducted at the Green Hospital of the Scripps Clinic and Research Foundation's Pheresis Unit on a Fenwall  
5 CS 3000 Blood Cell Separator according to the manufacturer's instructions for isolating lymphopheresed blood products. The resultant pellet was resuspended in Earle's medium (Flow Laboratories, Inc.) and the number of lymphocytes in the PBL cell  
10 suspension was determined.

## 2. Reconstitution of SCID Mice

Resuspended PBL prepared in Example 1 diluted to selected concentrations depending on the experimental  
15 design were injected intraperitoneally (i.p.) in C.B-17-scid/scid (SCID) mice (day 0). PBL were usually injected into SCID mice within 2-3 hours, and never more than 6 hours, after blood drawing. The range of PBL concentrations tested went from  $2.6 \times 10^6$   
20 up to  $50 \times 10^6$  cells/ml. The preferred PBL cell concentration was determined to be  $50 \times 10^6$  cells/ml. The SCID mice used for reconstitution experiments received the PBL injections at 2-3 months of age after being tested and determined not to be leaky.

25

## 3. Evaluation of Reconstituted Human Immune System in hu-PBL-SCID Mice

The results presented herein were compiled from 12 experiments involving more than 1100 SCID mice  
30 populated with PBL derived from 14 healthy human donors. SCID mice reconstituted with human PBL are designated hu-PBL-SCID in practicing this invention.

### A. Human IgG Levels in hu-PBL-SCID Mouse Sera

35 Initial experiments focused on measuring

human IgG serum levels in hu-PBL-SCID mice to ascertain the optimal number of human PBL necessary to successfully reconstitute a human immune system. Four of 15 SCID mice injected with  $2.6 \times 10^6$  PBL, 8 of 15 mice injected with  $5.2 \times 10^6$  PBL, and 15 of 15 SCID mice injected with  $10.5 \times 10^6$  PBL had increasing human IgG serum levels. Thirty days after PBL transfer, the mean human IgG serum level of these three groups were 740, 602 , and 1,464  $\mu\text{g/ml}$ , respectively. The 18 remaining hu-PBL-SCID mice receiving the smaller inocula had very low human IgG serum levels (2-26  $\mu\text{g/ml}$ ).

For a more complete analysis, SCID mice were then injected with  $15 \times 10^6$  PBL. This amount of cells was chosen for the analysis as it was consistently associated with peak serum levels of human IgG higher than 2,000  $\mu\text{g/ml}$  with a cell aliquot size that permitted large groups of SCID mice to be derived from the same donor. The mean human IgG serum level in hu-PBL-SCID mice increased for the first 2 months to a peak of approximately 3,500  $\mu\text{g/ml}$  as shown in Table 1 below, a level approximately 1/5 to 1/2 that of a normal human donor (normal human IgG serum level 7,000-17,000  $\mu\text{g/ml}$ ). In the few mice surviving beyond 2 months, human IgG serum levels decreased over the next year to a level approximately 1/10 that of the peak, but were still detectable for as long as two years as shown in Figure 1.



Table 1

Mean human IgG [ $\mu\text{g/ml}$ ] serum levels in 145 hu-PBL-SCID mice<sup>1</sup>

Group	D15	D30	D60	D90	D120	D150	D180	D270	D365
EBV+									
m <sup>2</sup>	1658	3843	4437	1768	2022	501	298	n.d. <sup>3</sup>	n.d.
sd <sup>4</sup>	1166	1549	2810	2126	2636	705	419	n.d.	n.d.
n <sup>5</sup>	59	67	35	3	3	2	2	n.d.	n.d.
EBV-									
m	1404	1820	2723	2621	1655	1452	968	618	322
sd	359	592	1375	1191	1211	1154	890	670	390
n	11	35	31	23	19	19	17	17	20

<sup>1</sup> SCID mice were populated with  $15 \times 10^6$  PBL, either from 3 donors (4 experiments) with (EBV<sup>+</sup> group), or one donor without (EBV<sup>-</sup> group) detectable serum anti-EBV antibody.

<sup>2</sup> mean IgG serum level [ $\mu\text{g/ml}$ ].

<sup>3</sup> not done (no mice alive).

<sup>4</sup> standard deviation within the group.

<sup>5</sup> number of hu-PBL-SCID mice tested.

The serum levels of human IgG in SCID mice populated with  $15 \times 10^6$  PBL from our EBV<sup>-</sup> donor were comparable to those of the Epstein-Barr Virus (EBV)<sup>+</sup> recipients at 15 days post PBL transfer, but were only 50% at day 30, and at peak levels on day 60 were only 60% that of the EBV<sup>+</sup> hu-PBL-SCID mice (Table 1).

Since irradiation of the SCID recipient improved reconstitution by isogeneic normal mouse stem cells as described by Fulop et al., J. Immunol., 136:4438-4443 (1986), whether such a manipulation prior to PBL transfer would enhance the human reconstitution in this model was investigated. To eliminate development of human B cell lymphoproliferative diseases, cells were transferred from an EBV<sup>-</sup> donor. Briefly, SCID mice irradiated to 250 rads proved to be better recipients of human B cell engraftment, as demonstrated by significantly ( $p < 0.05$ ) higher human IgG serum levels during the first 30 days post-PBL transfer than their non-irradiated counterparts receiving the same number of cells from the same PBL aliquot as shown in Figure 1. This difference disappeared by day 60 (Figure 1).

PBL that had been kept frozen in liquid nitrogen, thawed, adjusted to a number of viable cells ( $> 90\%$  of the total lymphoid cell population) that matched that of the original number of transferred PBL cells, and then transferred into SCID mice showed a significantly slower increase of recipient Ig serum levels ( $p < 0.001$  at day 15 and day 30) with a peak at around day 90 (Figure 1).

Manipulations of the recipient or the cells described above affected the timing, but not the magnitude, of human IgG formation. Human IgG, among the groups of mice that were either irradiated, injected with cells kept frozen, or controls, reached

similar maximal levels (2443, 2547, and 2723  $\mu\text{g/ml}$ , respectively) as shown in Figure 1.

B. Human IgM Serum Levels in hu-PBL-SCID Mice

5 IgG and IgM levels of 159 sera from hu-PBL-SCID mice populated with  $15 \times 10^6$  PBL were compared. The ratio of IgM to IgG mean serum levels in hu-PBL-SCID mice were 2.5-4.0 times lower than those in the respective three donors. The ratio of  
10 hu-PBL-SCID mice IgM over IgG serum levels increased in parallel with IgG serum levels (a linear regression analysis between these two parameters generated a positive slope of 0.027, with a correlation  
15 coefficient of 0.72). Human IgM peak levels in individual hu-PBL-SCID mice surviving for extended time periods were generally recorded between day 60 and 90, and were highly variable.

C. Anti-Tetanus Toxoid (TT) Antibody Serum Levels

20 The question of whether the higher reconstitution level achieved by the methods described above might lead to more consistent transfer of PBL expressing a specific antibody, such as anti-tetanus  
25 toxoid (TT), was addressed. A previously described PBL transfer method resulted in only 3 of 14 SCID mice populated with  $10-50 \times 10^6$  PBL from tetanus toxoid immune donors had detectable anti-TT antibody serum  
30 levels. To test whether an improvement would be achieved with methods as practiced in this invention, PBL from donors who were not exposed to TT for more than one year were evaluated.

The results of these experiments performed as described in Example 3A showed that all 42 hu-PBL-SCID  
35 mice populated with  $15 \times 10^6$  PBL from three different

donors (not boosted with TT for more than one year) had detectable levels ( $>0.008$  IU/ml) of anti-TT antibody 30 days post PBL transfer. Significant variability was observed from one hu-PBL-SCID mouse to another; the mean values of these three hu-PBL-SCID mouse groups were  $2.09 \pm 1.16$  ( $n=31$ ),  $0.78 \pm 0.84$  ( $n=4$ ), and  $0.21 \pm 0.16$  ( $n=7$ ) IU/ml. When normalized for the IgG serum level of the respective donors, these three groups of hu-PBL-SCID mice had corrected mean levels of anti-TT antibody of 11.19, 1.15, and 0.26 IU/ml (the respective serum levels of the human donors were 3.40, 0.63, and 0.35 IU/ml).

D. High Resolution Electrophoresis (HRE) of hu-PBL-SCID Mouse Sera

As a rough determination of the diversity of the human Ig produced in the model, HRE was performed on sera derived from 115 hu-PBL-SCID mice. Only 11 of 62 SCID mice populated with  $15$  or  $50 \times 10^6$  PBL from EBV<sup>+</sup> donors had electrophoretically restricted serum immunoglobulin migration pattern (mIg) before day 30. A long term analysis involving 127 sera from 53 SCID mice populated with  $15 \times 10^6$  PBL showed 16 of 25 EBV<sup>-</sup> hu-PBL-SCID mice, and 16 of 28 EBV<sup>+</sup> hu-PBL-SCID mice developing mIg. No EBV<sup>-</sup> hu-PBL-SCID mice had mIg prior to day 60, while 14 EBV<sup>+</sup> hu-PBL-SCID mice had mIg at day 43. Seventeen of 32 mIg<sup>+</sup> hu-PBL-SCID mice had multiple mIg at HRE, with comparable frequencies in recipients of EBV<sup>+</sup> and EBV<sup>-</sup> cells. mIg in EBV<sup>-</sup> hu-PBL-SCID mice differed from mIg in EBV<sup>+</sup> hu-PBL-SCID mice in their lower intensity, the absence of associated lymphoproliferative disease, and their high proportion of lambda light chain utilization (ten of 18 EBV<sup>-</sup> versus 2 of 9 EBV<sup>+</sup> mIg analyzed).

#### E. Survival of hu-PBL-SCID Mice

The presence of mIg was accompanied by human lymphoproliferative diseases in virtually all autopsied SCID recipients of PBL from EBV<sup>+</sup> donors.

5 These lymphoproliferative diseases were associated with the high mortality of EBV<sup>+</sup> hu-PBL-SCID mice. The fifty percent mortality of such reconstituted mice was at about 60 days.

10 About 40% of hu-PBL-SCID mice populated with  $15 \times 10^6$  PBL from an EBV<sup>-</sup> donor died between 30 and 170 days, although they never developed the large lymphomatous masses generally seen at autopsy of EBV<sup>+</sup> hu-PBL-SCID mice. These EBV<sup>-</sup> hu-PBL-SCID mice had clinical signs suggesting GvH disease (scruffy hair, 15 hunch back, anemia, and some developed generalized oedema). Investigations of the possible cause for this mortality indicated that human IgG serum levels at day 30 (reflecting functional human B cell reconstitution in SCID mice) were significantly higher 20 in mice that died prior to 170 days versus those surviving for more than 365 days.

#### F. Quantitation of Human Cells in hu-PBL-SCID Mouse Organs by Dot Blot Hybridization

25 The clinical and paraclinical signs described above are compatible with a pattern of GvH disease. GvH disease is in part mediated through the invasion of host tissue organs by cells originating from the graft. To evaluate the possible presence of 30 human cells in hu-PBL-SCID mouse organs, and to assess their proportion within each individual organ, dot blot analysis was performed using a human-specific ALU repeat family probe. The BLUR2 (human ALU repeat family probe), was subcloned in the Bam HI restriction 35 site of the pGEM-7Zf(+) vector (Promega Biotec,

Madison, WI) using standard procedures (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ED, Cold Spring Harbor Laboratory Press, New York, (1989)). The probe was labeled with  $^{32}\text{P}$  using the  
5 random hexamer priming method as described by Feinberg et al., Anal. Biochem., 132:6-13 (1983).  
Alternatively, radiolabeled  $^{35}\text{S}$ -RNA transcripts were generated after linearization of the plasmid construct with EcoRI or SacI, using T3 or T7 RNA polymerase,  
10 respectively. The transcription reaction conditions have been described by Pardoll et al., J. Exp. Med., 165:1624-1638 (1987). The  $^{32}\text{P}$  or  $^{35}\text{S}$  radiolabeled probes were extracted by phenol/chloroform, purified using a G-50 Sephadex column (Quick Spin column,  
15 Boehringer Mannheim Biochemicals, Indianapolis, IN), filtered through a 0.22  $\mu\text{m}$  filter, and stored in TE at  $-80^\circ\text{C}$  until use.

Dot blot analysis was performed as previously described by Duchosal et al., J. Immunol.,  
20 142:1737-1742 (1989) with a slight modification: the washing step was performed in 2x SSC-0.1% SDS at  $25^\circ\text{C}$  for one hour, in 2x SSC-0.1% SDS at  $67^\circ\text{C}$  for 30 minutes, and in 2x SSC-0.1% SDS at  $67^\circ\text{C}$  for 30 minutes. The radioactivity level of each dot was  
25 determined using a radioanalytic imaging apparatus (Ambis System, Inc., San Diego, CA), and filters were exposed 12-20 hours with XAR Kodak films. On each blot, control dots with known quantities of DNA from human PBL and from spleens of unmanipulated SCID mice  
30 were included.

Among 7 EBV $^{-}$ , and 2 EBV $^{+}$ -hu-PBL-SCID mice studied between 14 and 196 days post  $15 \times 10^6$  PBL transfer and without evidence of lymphoproliferative disease, DNA extracted from 6 livers and 5 kidneys  
35 yielded positive hybridization signals. Using a

radioanalytic imaging system, quantitative determination of the proportion of human cells in livers and kidneys never exceeded 2.6%. The presence of human cells was assessed in 4 of 6 DNA extracts from bone marrow and were in low proportion of the total cell population (less than 1.4%). Surprisingly, in the spleen, which was the lymphoid organ with the most dramatic increase in size after human PBL transfer, human cells did not exceed 8.4% of the total nucleated spleen cell population, and the highest percentage was generally recorded in hu-PBL-SCID mice more than 4 months after cell transfer.

G. Distribution of Human Cells Within Tissues and Organs of hu-PBL-SCID Mice Assessed by In Situ Hybridization

To determine the distribution pattern of human cells within the organs and further compare this with known models of GvH reactions, in situ hybridizations were performed using a specific human ALU repeat family probe as described in Example 3F. The in situ hybridization procedure was derived from previously described methods. See, Pardoll et al., supra; Jeong et al., J. Immunol., 140:2436-2441 (1988); and Mueller et al., J. Exp. Med., 167:1124-1136 (1988). Mice were sacrificed and autopsied when moribund or when scheduled for histologic analysis. Pieces of tissues were fixed in Bouin's, paraffin embedded, and 4 um sections were prepared.

Tissue sections were then deposited on precleaned glass slides, deparaffinized, and digested with 1 µg/ml of proteinase K (Beckmann) for 20 minutes. Following post-fixation in Bouin's for 20 minutes, the slides were acetylated in a 0.1 M triethanolamine, pH

8.0, solution containing 0.25% of acetic anhydride, and then transferred for 30 minutes into a solution containing 0.1 M Glycine. After dehydration in graded ethanol, the slides were air dried. Forty  $\mu$ l of hybridization solution containing 50% deionized formamide (Sigma, St. Louis, MO), 2x SSC, 10 mM DTT, 1 mg/ml of yeast tRNA (Boehringer Mannheim Biochemicals), 1 mg/ml of sheared salmon sperm DNA (Sigma), 2 mg/ml of nuclease-free BSA (BRL Life Technologies Inc.), and 16 x 10<sup>6</sup> cpm of <sup>35</sup>S radiolabeled BLUR2 riboprobe was placed over the tissue sections, and coverslip added. Following denaturation at 80°C for 3 minutes, the hybridization was carried out in an humidified chamber at 50°C for 14-16 hours. The slides were washed 3 times with 3 changes of 2x SSC, 50% formamide solution containing 0.2 M beta-mercaptoethanol (Sigma) at 54°C. Between these washing cycles, the tissue sections were digested with RNase A (100  $\mu$ g/ml, Worthington Biochemical), and RNase T1 (1 u/ml, BRL) in 2x SSC for 30 minutes at 37°C. The slides were dehydrated in graded concentrations of ethanol, air dried, coated with a nuclear emulsion (Ilford K5.D), exposed at 4°C for 9 days, and developed and fixed (D-19, Rapid fix, Kodak International Biotechnologies Inc.). After a final counterstain with Hematoxylin and Eosin, the slides were observed under a Nikon microscope equipped with an EGS filter which allows transmission and reflection fluorescent light (Nikon Inc.). Under these conditions, the black emulsion grains appeared turquoise.

Analysis included 6 unmanipulated SCID mice (3-15 months old), 57 EBV<sup>+</sup> hu-PBL-SCID mice derived from 5 donors, and 17 EBV<sup>-</sup> hu-PBL-SCID mice. Hu-PBL-SCID mice were populated with either 10 x 10<sup>6</sup> or, to better visualize areas of human cell infiltration in SCID



mouse organs,  $50 \times 10^6$  PBL. Human cell distribution was considered only in mice without obvious lymphoproliferative diseases. In the large majority of these mice, the primary organs (spleen, liver, genital organs, kidneys, and lungs) were examined, and in some individuals the pancreas, bone marrow, skin, and gut were also analyzed. No tissue from the unmanipulated SCID mice had positive cells at in situ hybridization. In contrast, human cells could be observed in all tissue samples from hu-PBL-SCID mice.

These human cells represented as a mean a low proportion of the total cell population, but again with a great individual variability. Human cells were generally detected in the primary non-lymphoid organs 30-60 days post PBL transfer, and the number of cells observed was generally higher in mice populated with high ( $50 \times 10^6$ ) vs. low ( $10 \times 10^6$ ) numbers of PBL. The distribution pattern of human cells was very constant in almost all animals and tissues studied regardless of their donor origin and cell number injected, and consisted mainly of perivascular adventitial infiltrates. In the liver, human cells were predominantly around biliary canaliculi and arterioles in portal spaces, with few cells detected in the sinusoids. In lungs, human cells were mainly distributed perivascularly and peribronchially, with a few cells detected in the septa. In kidney, human cells were mainly found perivascularly at the corticomedullary junction, around vessels ascending in the cortex, and periglomerularly. Male hu-PBL-SCID mice accumulated human cells in testicular interstitium, and this was observed with several donors both of male or female origin.

In the spleen 30 days after PBL transfer, human cells were found mainly in the white pulp, with very

few cells distributed in the red pulp. With time, the area infiltrated by human cells became progressively fibrotic so that at one year, the spleen was reduced to its original size. Perivascular fibrosis was  
5 mainly confined to the adventitia and was generally accompanied by mild stenosis without obstruction. Within this fibrosis, a few human cells persisted.

10 H. Human T and B Cell Distribution Evaluated by Immunohistochemistry

To further confirm the human origin of the cells in situ hybridized with the human ALU repeat family probe, and to evaluate the distribution of human B and T cells in hu-PBL-SCID mouse primary  
15 organs, immunohistochemistry on 2 unmanipulated SCID, 11 EBV<sup>+</sup>hu-PBL-SCID, and 6 EBV<sup>-</sup>hu-PBL-SCID mice populated with  $10\text{--}50 \times 10^6$  PBL. Tissue sections as prepared in Example 3G were deparaffinized through xylene washes. After a rinse for 10 minutes in  
20 distilled water, the sections were maintained for 1 hour at 37°C, and overnight at 4°C, with either a monoclonal mouse anti-human leukocyte common antigen (CD45, 2B11, and PD7/26, Dako Corp.), mouse anti-human T cell (UCHL1, Dako Corp.), mouse anti-human B cell  
25 (B1, Coulter Immunology), or polyclonal rabbit anti-human IgG or anti-human IgM (Dako Corp.), antibodies. Controls included incubation with non-immune mouse and rabbit sera. The sections were further processed for indirect immunoperoxidase  
30 visualization using diaminobenzidine (DAB) as the chromogen accordingly to manufacturer's instructions (Vectastain Elite ABC kit, Vector Laboratories) using as secondary antibodies either a horse anti-mouse, or a goat anti-rabbit IgG conjugated to avidin (Vector  
35 Laboratories).

No mice analyzed were leaky at time of sacrifice. No reactivity was observed with tissue sections from unmanipulated SCID mouse primary organs, as well as hu-PBL-SCID mice tissue sections from primary organs incubated with non-immune mouse and rabbit sera. The human cell origin of the positive cells with in situ hybridization as described in Example 3G was confirmed by their surface reactivity with antibody directed against human leukocyte common antigen. Human T cells were found in the center portion of the white pulp of the spleen at day 30 or later, and included the majority (>95%) of cells infiltrating tissues described above. Human B cells, defined by their reactivity with antibody against human CD20, human IgG, or human IgM, made up <5% of the human cells infiltrating most tissues. The only exceptions were spleens of hu-PBL-SCID mice at day 30 or later, where B cells were of variable proportion of the total human cell population (up to 40% in 2 hu-PBL-SCID mice) and clustered primarily at the periphery of the white pulp, and in the red pulp. The shape and Ig pattern staining of these B cells suggest that the majority were plasma cells.

4. Analysis of the Reconstituted Human Immune System in hu-PBL-SCID-Mice

The human immune system reconstituted in SCID mice using a transfer method that minimizes both the time and manipulations required to transfer PBL was characterized. This procedure, described above in Examples 1-3, permitted 27% of SCID mice to acquire significant human IgG serum levels when injected with as few as  $2.6 \times 10^6$  PBL, and 100% developed 2-5 g/l of human IgG in their sera when injected with  $15 \times 10^6$  PBL. These human IgG levels were previously

associated with higher numbers of PBL transferred, or such levels were lower with similar numbers of cells used. See, Mosier et al., Nature, 335:256-259 (1988) and Mosier, J. Clin. Immunol., 10:185-191 (1990).

5 This method employs relatively few cells to achieve a significant reconstitution of a human immune system, and will be particularly useful in studies of patients with low peripheral lymphocyte counts, such as AIDS or other lymphopenic patients, as well as in studies  
10 requiring large numbers of recipient mice.

The kinetics of human IgG serum levels in hu-PBL-SCID mice surviving more than 5 months after PBL transfer was similar to those of SCID mice populated with a method involving more manipulations  
15 of the transferred cells, and consisted of a dramatic increase during the first two months, followed by a decrease over many months without returning to zero. Low-dose irradiation of the mouse recipient appeared to significantly increase human IgG serum levels in  
20 hu-PBL-SCID mice during the first month post PBL transfer. This manipulation may, therefore, be useful for short-term studies requiring a highly reconstituted human immune system.

The majority of hu-PBL-SCID mice had relatively  
25 low serum levels of human IgM compared to IgG, an observation similar to that reported with SCID mice populated with heavily manipulated PBL. Interestingly, hu-PBL-SCID mice with the highest IgG serum levels also had the highest ratio of human IgM  
30 relative to IgG, suggesting a distribution of these two isotypes more similar to that of human donors in individuals showing the greatest B cell activity.

One characteristic of the hu-PBL-SCID murine models described previously appeared to be the limited  
35 number of human Ig-producing B cells in the recipients

as described by Saxon et al., J. Clin. Invest.,  
87:658-665 (1991). This conclusion was derived  
partially from the fact that a limited number of  
predominant bands (<30) was observed on isoelectric  
focusing of hu-PBL-SCID mouse sera, and also because  
only a minority of hu-PBL-SCID mice had anti-TT  
antibody serum levels when populated from PBL of  
donors with such levels.

In this invention, 100% of SCID mice populated  
with  $15 \times 10^6$  PBL from 3 donors with detectable  
anti-TT antibody serum levels, who had not been  
recently boosted, had such antibody. This observation  
was strongly indicative of the functionality of  
specific T-B cell cooperation for this  
thymus-dependent antigen in the model. Surprisingly,  
at 30 days post PBL transfer, two of three donor  
derived groups of hu-PBL-SCID mice had about 2-3 fold  
higher mean IgG anti-TT levels than in the respective  
human PBL donor sera when normalized for human IgG  
serum levels. This might result from a  
disproportionate concentration of anti-TT antibody-  
producing cells in the circulation and/or from  
activated xenoreactive T cells producing non-specific  
help to specific anti-TT antibody-producing B cells in  
these two hu-PBL-SCID mouse groups.

Human Ig diversity, assessed by a smeared Ig  
pattern at serum HRE, was generally observed during  
the first month post PBL transfer. A more extensive  
analysis performed at this time on two hu-PBL-SCID  
mouse sera revealed the presence of the four human IgG  
subclasses proportional to that of the respective  
human donors. PBL derived from donors previously  
infected with EBV produced more IgG than EBV<sup>-</sup> PBL  
between 1 and 2 months post PBL transfer. The earlier  
development of mIg in EBV<sup>+</sup> versus EBV<sup>-</sup> hu-PBL-SCID mice

was certainly one of the major reasons for this observation, although an underlying polyclonal B cell stimulation secondary to EBV presence, or individual donor variability, cannot be ruled out. Surprisingly, 5 EBV<sup>-</sup>hu-PBL-SCID mice developed mIg at the same frequency as EBV<sup>+</sup>hu-PBL-SCID mice, although later. All these mIgs were clearly of human origin because they were immunofixed with anti-human specific Ig antibodies and observed in SCID mice, the large 10 majority of which were not leaky at the time of analysis. The observation that the panel of mIg appearing in EBV<sup>-</sup>hu-PBL-SCID mice was composed almost equally of lambda and kappa light chains, whereas mIg from EBV<sup>+</sup>hu-PBL-SCID mice utilized kappa more than 15 three times as frequently as lambda light chain, might indicate that the mechanism controlling B cell production of mIg differed between EBV<sup>+</sup> and EBV<sup>-</sup> hu-PBL-SCID mice.

With EBV<sup>+</sup> donors, the present transfer method was 20 accompanied by a high incidence of fatal B cell lymphoproliferative diseases beginning in the second month which, for practical purposes, limited the period of meaningful serological observations. This incidence of lymphoproliferative diseases has been 25 reported earlier with much higher numbers of transferred cells as described by Mosier et al., Nature, 335:256-259 (1988).

SCID mice populated with EBV<sup>-</sup> donor PBL displayed a high proportion (>50%) of clinical signs compatible 30 with GvH disease, and had a high mortality (>40%) although not suffering from lymphoproliferative disease. The individual variability in reconstitution, globally assessed by the human IgG serum levels at day 30, led us to conclude that the 35 highly reconstituted SCID mice with high serum IgG

were prone to die earlier than the others.

The possibility of a human GvH mouse disease in SCID mice with high human lymphoid cell reconstitution was further investigated using methods to determine the presence, and distribution of human cell(s) (subsets) in the model. These analyses, performed on a time course basis in several individuals, also provided information concerning the kinetics of human cells in the model.

As early as 15 days after i.p. injection, human cells were readily apparent in the circulation of hu-PBL-SCID mice (in situ hybridization and FACS analysis). Very few human cells were present in parenchymal organs. At one month post PBL transfer, human T cells were detected in the perivascular areas of parenchymal organs, and human T and B cells were readily apparent in the spleen, where T cells predominated in the white pulp and human B cells in the red pulp as well as at the periphery of the white pulp. Interestingly, at this age 50% of the EBV hu-PBL-SCID mice were sick, and some started to die. The combination of clinical signs (present even when populated with PBL from an EBV donor) and human T cell distribution in many parenchymal organs (observed in hu-PBL-SCID mice derived with PBL from all donors) resulting in fibrosis of afflicted areas, indicated a GvH phenomenon, although other possibilities, such as viral infection and/or reactivation (which would have occurred in the majority of hu-PBL-SCID mice studied here), cannot be completely excluded. Previous reports indicated that hu-PBL-SCID mice do not have or have at most minimal GvH (Mosier et al., Nature, 335:256-259, 1988).

The observations presented herein definitively rule out the possibility of an underlying

lymphoproliferative disease secondary to EBV presence as being responsible for the sickness of the reconstituted animals. The differences in GvH expression may be explained by quantitative and/or qualitative variations in the human cell population engrafted in hu-PBL-SCID mice. The GvH disease probably influenced the mortality rate in EBV<sup>+</sup>hu-PBL-SCID mice, and explained the absence of lymphoproliferative disease recorded at autopsy of some sick EBV<sup>+</sup> hu-PBL-SCID mice.

However, as a global pattern, the anatomical reconstitution of a human immune system by PBL transferred in the SCID mouse is obviously incomplete. For example, no follicles were seen in the spleen and the lymph nodes were not enlarged. Human T and B cells were nevertheless histologically detectable for extended periods of time, an observation that could provide important insights for the use of this model in human infectious diseases (such as AIDS). Moreover, the likelihood that the xenotransplanted human lymphoid cell grafts reacted against the murine environment, and therefore were functional, could expand the use of this model for the study of human GvH, and for significant pre-transfer in vitro or direct in vivo immunization or other manipulations of the human immune system outside the human environment.

## 5. Contacting Human Immune Cells with Antigen

### A. In Vitro Methodology

#### (1) Immunization with HBc Antigen

The first set of experiments to evaluate the effect of in vitro exposure of PBLs to antigen on subsequent antibody production after injecting the PBL into SCID mice involved the use of hepatitis B core (HBc) antigen. This antigen was



chosen because it is a potent thymus-dependent, and  
-independent antigen as well as a very good immunogen  
(Milich et al., Science, 234:1398-1401, 1986). In  
addition, a spectrum of donors with no, low or high  
antibody titers for this antigen were available.

SCID mice were bred in the Scripps facility  
colony, in a specific pathogen-free environment, and  
tested for the presence of mouse Ig at 6-8 weeks of  
age. Leaky SCID mice were removed from the study, and  
the remaining SCID mice populated with PBL before they  
were 9 weeks old. HBc antigen was prepared as  
follows: E. coli bacteria (JM109 strain) were  
transformed with the pFS14 expression plasmid, induced  
with IPTG and lysed according to Stahl et al., Proc.  
Natl. Acad. Sci. USA, 79:1606-1610 (1982). Following  
DNaseI digestion, the protein was further purified by  
selective centrifugation, gel filtration  
(BIO-Gel-A-50m, Biorad), and finally hydroxylapatite  
chromatography (Bio-Gel-HT, Biorad).

Lymphophereses as described in Example 1 were  
performed at Scripps Clinic and Research Foundation  
from donors giving their informed consent. The cells  
were washed in Earles medium (Flow Laboratories, Inc.)  
to eliminate human plasma, highly toxic when injected  
i.p. into SCID mice, but no attempt was made to  
eliminate the erythrocytes and platelet contaminants.  
Hu-PBL-SCID mice, previously immunized with HBc  
antigen, were i.p. injected (day 0) with  $50 \times 10^6$  PBL  
previously maintained for 4 hours at a concentration  
of  $4 \times 10^6$ /ml (RPMI-10% FCS was used to dilute the  
lymphopheresis product) with 2 ug/ml of purified  
recombinant HBc in an humidified incubator at 37°C  
with 7% CO<sub>2</sub>. These mice were subsequently injected  
subcutaneously (s.c.) with 10 µg of HBc in CFA at the  
base of the tail on day 2, and with the same dose of

antigen s.c. in saline on day 22 or 23. Control groups were processed in parallel and consisted of SCID mice populated with  $50 \times 10^6$  PBL incubated without antigen, injected on day 2 with CFA, and on day 22/23 with saline only. Additional experimental groups were evaluated as described below in the results.

Hu-PBL-SCID mice were bled sequentially, and human IgG serum levels determined as described by Duchosal et al., J. Exp. Med., 172:985-988 (1990). IgG anti-HBc titers were determined by indirect ELISA. Microtiter flat-bottom plates (Costar) were coated with 0.5 ug/ml of purified recombinant HBc in PBS overnight at 4°C. Non-specific binding was prevented by maintenance with PBS-5% non-fat dairy milk. Serial dilutions of collected sera starting at 1/200 from hu-PBL-SCID mice, the respective donor, a donor known for high IgG anti-HBc activity (standard), as well as from humans with no history of hepatitis, were further maintained in duplicate. After one hour maintenance at 37°C, the specific human IgG anti-HBc was revealed using a peroxidase labeled, affinity-purified, goat anti-human IgG (Cappel), followed by the addition of 0-phenylene-diamine (Sigma). The reaction was stopped by the addition of  $H_2SO_4$ , and OD determined at 492 nm. After each step, several washes with PBS-0.05% tween were performed. The titer corresponding to an OD of twice that of normal negative donor serum at lowest dilution was recorded (absolute value). The human specificity of these assays were confirmed by its non-reactivity with sera from unmanipulated SCID, C57BL/6, and BALB/c mice, non-immunized and immunized with HBc. Normalized titers were calculated as follows: absolute titer x [serum IgG level (donor)/serum IgG level (hu-PBL-SCID mouse)].

5 Normalization allowed unambiguous differentiation between passive and active changes of the antibody tested, and therefore a reliable assessment of the putative immune response within a given mouse with time or between groups of hu-PBL-SCID mice derived from a common donor. The results of these assays are discussed below.

10 All twelve non-immunized hu-PBL-SCID mice, populated with cells from a donor (#1) without detectable anti-HBc antibody levels, lacked such antibody. In contrast, three of 12 hu-PBL-SCID mice receiving similar cells and immunized with a purified recombinant HBc antigen displayed only transiently detectable, low, human IgM anti-HBc titers at day 15 (1/50, 1/50, and 1/475). Only one of these three hu-PBL-SCID mice had detectable anti-HBc human antibody titers (1/90) one week after booster immunization, again limited to the IgM isotype. These responses were definitely of human origin because the detecting antibody was human specific and the respective SCID mice were not leaky at the time of testing.

25 When PBL from a donor (#2) with low serum IgG anti-HBc titer (1/320) were used, two of 10 immunized hu-PBL-SCID mice showed IgG anti-HBc responses, with 16 non-immunized controls remaining below the threshold of detectability. This on/off phenomenon of the immune response seen when donors have little or no antibody against the immunizing antigen probably results from the fact that not all mice received adequate quantities of the right combination of B and/or T cells specific for the immunizing antigen. Interestingly, the antibody levels of the two responding hu-PBL-SCID mice from donor #2 were of far greater magnitude than that of the donor (2-3 log

difference). This model can, therefore, serve as an in vivo subcloning system to select, for further manipulations, immunized hu-PBL-SCID mice with specific antibody levels much higher than those of the respective human PBL donor.

The transfer of PBL to SCID mice from a patient (#3) recently infected with hepatitis B virus (HBV), with a higher IgG anti-HBc titer (1/7,700), resulted in the appearance of specific IgG antibodies in 100% of the control non-immunized recipients. These anti-HBc antibodies were actively produced in hu-PBL-SCID mice because they were not detected early (at day 2), and were maintained much longer than would passively transferred antibody. The median titers (normalized and absolute) of this group were below those of the donor. Decreased T-B cell cooperation for this antigen in these mice, and production of human anti-mouse antibodies secondary to GVH, could explain the decrease in absolute and even more in normalized IgG anti-HBc titers with time, respectively. All 11 hu-PBL-SCID mice populated with cells from this donor, and immunized with HBc, responded with increased IgG anti-HBc titers over 10 times higher than those in the control non-immunized group. In contrast to the 2 responder mice from donor #2, these mice showed no increase in IgG anti-HBc titers after booster injection at three weeks, and the magnitude of their anti-HBc antibody response was lower. The specificity of the anti-HBc antibody response was indicated by the fact that the mean antibody levels against another protein antigen (TT) of non-immunized and HBc immunized hu-PBL-SCID mice groups were similar.

The results are summarized as follows: a) specific antibody production in the absence of

antigenic exposure of cells or recipients is observed only when the donor has intermediate or higher levels of IgG anti-HBc; b) three of 12 HBc-immunized hu-PBL-SCID mice derived from a donor with no specific serum titer display low human anti-HBc antibody titers peaking at day 15 limited to the IgM isotype; c) the titer of specific IgG in the donor is correlated with the proportion of responder mice in the group rather than with the level of the immune response in an animal. The level of this response can be of far greater magnitude than the level of the donor; and d) the IgG anti-HBc response was specific.

(2) Immunization with HBc-Pre-S1 and Pre-S2 Antigens

Three connected surface glycoproteins, called large, middle, and major proteins, are present at the surface of the HBV. These proteins are produced by three alternate translation initiation sites defining the pre-S1, pre-S2, and S regions, respectively (Tiollais et al., Nature, 317:489-495, 1985). The proteins derived from these regions are far less immunogenic than the HBc antigen (Milich et al., Science, 234:1938-1401, 1986).

First, experiments were designed to test whether hu-PBL-SCID mice, derived from a donor (#3) with a low anti-pre-S1 antibody titer, could be immunized with a pre-S1 antigen. A purified recombinant protein antigen containing the full length sequence of the HBc protein and an immunogenic sequence from the pre-S1 region (HBc-pre-S1 antigen) was used for these experiments. This construct was designed to increase the immunogenicity of the pre-S1 protein due to the carrier effect of HBc antigen (Milich et al., Nature, 329:547-549, 1987).

The plasmid HBc-pre-S1 is composed of the

full-length sequence of the gene for the HBc protein with an upstream sequence corresponding to the pre-S1 12-47 amino-acid region [Tiollais et al., supra; Valenzeula et al., Nature, 280:815-819 (1979); Galiber et al., Nature, 281:646-650 (1979); and Pasek et al., Nature, 282:575-579 (1979)] inserted in the pKK-223 expression vector (Pharmacia LKB). Recombinant HBc-pre-S1 protein was purified as described by Milich et al., Nature, 329:547 (1987), for HBc. The pre-S2 peptide is a mixture of amino-acid sequences 148-174 (T cell epitope) linked to 131-143 (B cell epitopes) of the adw and (ayw, adr) subtypes of the pre-S2 region.

SCID mice were populated with PBL as described in Examples 2 and 3, immunized, and the control group processed as described above for Hbc. The following modifications were made to the immunization protocol: SCID mice were populated with PBL in vitro incubated either with 3  $\mu$ g/ml of HBc-pre-S1 antigen or 20  $\mu$ g/ml of pre-S2 peptide antigen, and injected at day 2 and 22 with 20  $\mu$ g of HBC-pre-S1 antigen, or 50  $\mu$ g of pre-S2 peptide antigen, respectively. IgG anti-pre-S1, and IgG anti-pre-S2 titers were determined by ELISA, as described for HBc above, with the following modification: microtiter plates were coated overnight at 37°C with 10  $\mu$ g/ml of a mixture of peptides corresponding to the adw2, and ayw subtypes of either the HBV amino-acid sequence 12-47 (pre-S1), or 120-145 (pre-S2), and the starting serum dilutions were 1/50. The specificity of the assay was assessed as described above for HBc.

The immunogenicity of the recombinant HBc-pre-S1 protein antigen was established by the fact that one hundred percent of hu-PBL-SCID mice derived from donor #3, and immunized with this antigen mounted a brisk

IgG antibody response against HBc. These mice immunized with HBc-pre-S1 had significantly higher IgG anti-pre-S1 titers than controls, with peak median normalized IgG anti-pre-S1 titer at day 15 corresponding to a 4-fold increase relative to the donor, with one individual having an 11-fold increase. No correlation could be observed between the level of IgG anti-HBc, and IgG anti-pre-S1 response within an individual. Among the control, non-immunized, donor #3 derived hu-PBL-SCID mice, which had very low median serum IgG anti-pre-S1 titers, one mouse showed an increased titer at day 36 to an absolute and normalized value of about twice that of the donor. This individual variability, which could be exploited in defining the role of a particular antibody without requiring immunization of the recipient with the relevant antigen, confirmed the validity of using large groups of hu-PBL-SCID mice to assess the effect of particular manipulations on the reconstituted human immune system.

The second question addressed was the feasibility of using this system for peptide immunization. The immune response of hu-PBL-SCID mice was tested, where the mice were immunized with a peptide containing 2 overlapping B cell epitopes, and a T cell epitope of the pre-S2 region (pre-S2 peptide). Non-immunized hu-PBL-SCID mice derived from donor #3 (who had intermediate levels of IgG anti-pre-S2 titers) have generally such titers in their sera again with individual variability. Nevertheless hu-PBL-SCID mice immunized with the pre-S2 peptide had median normalized IgG antibody serum levels between day 15 and day 29 more than twice, with five of 12 individuals more than 3 times, those of the controls.

(3) Immunization with Tetanus Toxoid  
Antigen

Tetanus toxoid (TT), a thymus-dependent antigen, was utilized in the model because a) the kinetics of human antibody responses to a challenge with this antigen are well characterized and provide a reference for consideration of the antibody response in the hu-PBL-SCID mouse model; b) the time between the last contact with this antigen (immunization) and the PBL transfer to the SCID mouse can be accurately determined.

For the experiments, two donors were chosen: one (#4) immune to this antigen, but not boosted with TT for 17 years, and one immune donor (#5), boosted 6 days prior to PBL transfer. Immunized and control groups of hu-PBL-SCID mice were handled as described above for HBC with the following modification: in the immunized group, PBL were maintained in vitro with 15  $\mu\text{g/ml}$  of TT antigen (Wellcome), and 50  $\mu\text{g}$  of TT were injected into mice at day 2, and 23. IgG anti-TT levels were determined as described below with the exception that an Fc-specific mouse-anti-human anti-Ig antibody was used to detect anti-TT antibodies that immunoreacted with the TT-coated wells. The threshold of the assay was 0.003 IU/ml.

The results of these experiments are shown in Figures 2A through 2E. One hundred percent of non-immunized hu-PBL-SCID mice derived from these two donors had detectable IgG anti-TT levels (Figures 2A, 2C). Both absolute and normalized IgG anti-TT levels in these two groups decreased with time, as was generally observed with the previously described antigens. Hu-PBL-SCID mice derived from donor #5 (Figure 2C) had higher serum IgG anti-TT levels than those derived from donor #4 (Figure 2A),



but the ratio of the median normalized levels of #5 immunized versus #4 immunized hu-PBL-SCID mice groups diminished with time. This suggests that human PBL stimulated in the donor continued their immune response in the SCID mouse, but that this declined with time.

After day 15, TT immunized hu-PBL-SCID mice derived from donors #4 (Figure 2B) and #5 (Figure 2D) had median normalized serum IgG anti-TT levels 8-60 and 3-12 times higher than their corresponding time-matched non-immunized controls, respectively. Hu-PBL-SCID mice populated with PBL from donor #4 had median normalized IgG anti-TT levels approximately 9 times higher, with some individuals having up to a 40- to 60- fold increase, over the level recorded in the donor which level at day 0 was 0.65 and at day 37 at 7.60 [IU/ml]. These increases are comparable to those reported for serum IgG anti-TT levels in humans 2 weeks or later after booster immunization (Farzad et al., J. Immunol. Methods, 87:119-125, 1986). The difference between the normalized median serum IgG anti-TT antibody levels of the two immunized groups diminished with time so that they became similar two weeks after the last booster immunization (Figures 2B, 2D). This means that the advantage of in vivo immunization of the donor 6 days prior to PBL transfer on the specific antibody production in this immunized chimeric model appears transient.

For the ELISA assays for the detection of the subclasses of human IgG anti-TT, microtiter plates (Immulon) were coated with TT (Wellcome) in carbonate buffer, and non-specific binding prevented by incubation with PBS-0.1% BSA. Donor or hu-PBL-SCID mouse serum dilutions were applied in duplicate, and maintained for 1 hour at 37°C and 12 hours at 4°C.

After several washes with PBS-0.05% tween, monoclonal mouse anti-human IgG1 (HP6001), IgG2 (HP6014), IgG3 (HP6050), or IgG4 (HP6025) were added at low dilution to assure saturation, and maintained for 1 hour at 37°C, and 12 hours at 4°C. These monoclonal antibodies have been characterized extensively with regard to their allotypic specificity (Reimer et al., Hybridoma, 3:263-275, 1984), and shown to bind specifically to a large panel of appropriate myeloma derived human IgG subclass standards (Rock et al., N. Engl. J. Med., 320:1463-1469, 1989). After several washes with PBS-tween, a goat anti-mouse kappa chain antibody conjugated to alkaline phosphatase (Caltag Lab.) was applied. After final washes with PBS-tween, the O.D. at 410 nm was read after p-nitrophenyl phosphate substrate (Sigma) addition. The O.D. obtained after a similar procedure, but without the addition of mouse anti-human IgG subclass antibody was subtracted.

The ELISA results show that IgG anti-TT response in human is highly subclass specific (80-90% IgG1) [Yount et al., J. Exp. Med., 127:633-646 (1968); and Seppala et al., Eur. J. Immunol., 14:868-875 (1984)]. This IgG1 skewed usage in the IgG anti-TT response is also observed in non-immunized hu-PBL-SCID mice populated with PBL from donor #4. Moreover the increase in IgG anti-TT levels, observed when the PBL have been stimulated in the donor just before and/or after their transfer into the mouse, is mainly secondary to an increase in IgG1 anti-TT serum reactivity.

The conclusions reached using the above-described panel of donors are the following: a) Significant human IgG anti-TT responses can be obtained in 100% of TT-immunized chimeric animals, even when the last

boost of the donor with the antigen was more than 20 years ago, and when the donor has no significant serum IgG anti-TT; b) Boosting of the donor with TT six days prior to cell transfer increased the IgG anti-TT  
5 levels of non-immunized hu-PBL-SCID mice, but did not seem to increase dramatically the specific response of immunized animals over those similarly treated but receiving cells from an immune, non-recently boosted donor; and c) The IgG anti-TT response in TT-immunized  
10 hu-PBL-SCID mice is generally, as observed in humans, IgG1 subclass restricted.

An additional set of experiments were performed to compare the difference between levels of human IgG and anti-TT in hu-PBL-SCID mice that had received  
15 injections of PBL exposed to the TT antigen in vitro to those that had not been exposed. The PBL were isolated from a donor who had an anti-TT titer of 0.865 IU/ml as measured by ELISA using a Fc-specific anti-Ig antibody as described above. The isolated PBL  
20 were then exposed to 15  $\mu$ g/ml TT for four hours immediately prior to injection into SCID mice as described in Examples 2 and 5. A duplicate amount of PBL was maintained in saline for the same period of time as a control. At day 0,  $50 \times 10^6$  PBL,  
25 antigen-exposed and saline-exposed, were injected into two groups of SCID mice as described in Example 2. At day 3, in the group of ten mice that received the TT-exposed PBL received a booster of TT (50  $\mu$ g/ml) in CFA followed by a second booster at day 23 of TT (50  
30  $\mu$ g/ml) in saline. The control group of ten mice received similar injections that lacked the TT antigen. Serum samples were collected from the mice at day 3, 15, 23, 30, 37 and 49. The amount of human Ig as well as specific anti-TT antibody were measured  
35 as described above.

The results of these experiments are shown below in Table 2 and Table 3. Table 2 contains the levels of non-specific human Ig and specific anti-TT antibody for mice that received PBL exposed to TT in vitro and boosters of TT. Table 3 contains the levels of the control animals. The determined levels of both non-specific human Ig in milligrams/liter (mg/L) and specific anti-TT antibody in IU/ml are presented in a column format based on the days the serum was collected. The ten mice injected in each group are numerically designated.

Table 2

	Mouse	Day 3		Day 15		Day 23		Day 30		Day 37		Day 49	
		HGG	$\alpha$ TT	HGG	$\alpha$ TT	HGG	$\alpha$ TT	HGG	$\alpha$ TT	HGG	$\alpha$ TT	HGG	$\alpha$ TT
15	692	80	neg <sup>1-10</sup>	233	11.1	4210	18.0	7206	18.2	9392	22.2	N.A.	N.A.
	693	70	neg <sup>1-10</sup>	1820	9.9	2813	21.9	4625	30.9	6688	40.4	N.A.	N.A.
20	695	66	neg <sup>1-10</sup>	1343	9.6	2663	14.8	4200	16.1	5336	10.3	DB day 43	
	696	67	neg <sup>1-10</sup>	2388	13.9	3175	24.2	5153	27.8	5744	24.2	DB day 43	
	697	89	neg <sup>1-10</sup>	2343	9.4	3150	19.2	5338	27.3	7256	32.5	DB day 43	
	700	43	neg <sup>1-10</sup>	238	2.4	398	5.9	548	5.4	780	8.7	799	7.0
25	701	75	neg <sup>1-10</sup>	1586	10.8	2350	22.9	3588	35.1	N.A.	N.A.	N.A.	N.A.
	702	60	neg <sup>1-10</sup>	1010	4.1	1781	9.5	3515	8.6	2623	1.3	2052	1.8
	705	73	neg <sup>1-10</sup>	1544	8.8	2688	14.0	3768	11.6	2844	9.6	DB day 44	
	706	71	neg <sup>-10</sup>	1648	9.3	2725	11.5	4513	17.7	5696	31.3	DB day 44	
30	Mean	69	neg <sup>1-10</sup>	1625	8.9	2595	16.2	4243	19.9	5151	20.0	1426	4.4
	SD	12		669	3.3	993	6.1	1693	10.0	2652	13.2	886	3.7
	#	10		10		10		10		9		2	

HGG = mg/L (Human gamma globulin, i.e., non-specific Ig)

$\alpha$ TT = IU/ml

DB day = mouse dead by the day indicated.

N.A. = sample not available because the mouse expired.

Table 3

	Mouse	Day 3		Day 15		Day 23		Day 30		Day 37		Day 49	
		HGG	$\alpha$ TT	HGG	$\alpha$ TT	HGG	$\alpha$ TT	HGG	$\alpha$ TT	HGG	$\alpha$ TT	HGG	$\alpha$ TT
5	678	68	.004	1731	.01	2214	.04	3595	.68	5110	.61	DB day 43	
	679	111	.005	1844	.02	2988	.14	4515	.47	DB day 35		N.A.	N.A.
	680	81	.004	1103	.02	1849	.10	2905	1.10	4930	1.96	6608	.78
	681	109	.005	1725	.02	225	.07	2585	.11	3635	.14	DB day 45	
10	682	45	.004	473	.00	1450	.01	2048	.14	3335	.36	2560	.08
	683	50	.004	941	.09	1303	.20	2245	.36	2265	.31	1944	.14
	686	71	.005	1368	.02	2428	.31	3640	.58	4730	.67	DB day 48	
	687	66	.004	1425	.03	2426	.19	5895	.57	DB day 33		N.A.	N.A.
	689	67	.004	1725	.08	2170	.16	4050	.65	3545	.52	DB day 43	
15	690	62	.004	1044	.00	1869	.01	3120	.04	2130	.05	1750	.01
	Mean	73	.004	1338	.03	3092	.12	3460	.47	3760	.58	3216	.25
	SD	22		443	.03	495	.10	1160	.32	1084	.60	2288	.36
	#	10		10		10		10		8		4	

The legend to Table 3 is the same as the legend to Table 2.

The results show that at day 3, negligible anti-TT antibody was produced by both groups and non-specific Ig levels were also low. However, by day 15, while the levels of non-specific Ig rose dramatically and comparably for both groups, the mice in Table 2 where PBLs were exposed to TT in vitro exhibited a marked rise in anti-TT antibody levels. The levels in this group of mice continued to rise throughout the time course evaluated and in some mice levels of anti-TT antibody reach 40.4 IU/ml. In addition, by day 30 the non-specific Ig levels were significantly higher and surpassing 6 mg/L in some of the mice receiving TT-exposed PBL and TT boosters compared to those that received saline-exposed PBL and saline boosters. Thus, a effective human immune response was mounted in mice reconstituted with PBL

that had been exposed in vitro to TT and subsequently boosted with TT compared to no response in mice reconstituted with PBL that were exposed in vitro to saline and subsequently boosted with saline.

5

#### B. In Vivo Methodology

Additional experiments were performed to determine the effectiveness of antibody production from PBL that were not exposed to TT in vitro prior to injection into SCID mice but were injected i.p. with TT simultaneously with 50  $\mu$ g/ml TT (equivalent to the day 2 injection scheme described in Example 5A) into SCID mice. The PBLs were isolated from a donor who had not been boosted with TT within a year and 50 X 10<sup>6</sup> cells were injected as described in Examples 2 and 5. The mice received a subsequent i.p. injection of TT (50  $\mu$ g/ml) at day 15. Serum levels of anti-TT in the SCID mice were measured at day 15, 21, 28, 35 and 42 after PBL injection. The anti-TT serum levels were measured as described in Example 5A.

The results obtained from mice injected with TT simultaneously with PBL that had not been exposed to TT in vitro were compared against control mice that did not receive any TT antigen at all, against mice that received PBL exposed to TT in vitro for four hours with 15  $\mu$ g/ml TT and subsequent injections of TT (50  $\mu$ g/ml) subcutaneously at days 0 and 15, and against mice that received PBL exposed to TT in vitro for four hours with 15  $\mu$ g/ml TT and subsequent injections of TT (50  $\mu$ g/ml) i.p. at days 0 and 15. For the day 0 s.c. injections, TT was admixed with CFA while for the day 15 injections, TT was admixed in saline. For the i.p. injections, TT was admixed in saline for both injections.

The results showed that while the in vitro

exposure to antigen followed by i.p injections of TT provided the most optimal production of anti-TT antibody, injecting PBL that had not been exposed in vitro to TT but were injected with TT simultaneously, i.e., in vivo, resulted in levels of anti-TT antibody that were comparable to those seen with mice injected with PBL exposed to TT and receiving the subcutaneous TT injections. Thus, the in vivo exposure to TT at times substantially simultaneously with the introduction of PBL was effective at promoting anti-TT antibody production by PBL.

Thus, hu-PBL-SCID mice can be immunized with protein and peptide antigens to mount a low primary (IgM) or a large secondary (IgG) antibody response. The latter occurs even when the human donor has a low level of antibody against the desired antigen and/or has not been exposed to the antigen for an extended period of time. The assessment of the functionality of the transferred human immune system in SCID mice has evident consequences in the study of the model. Such PBL derived from patients with localized or generalized autoimmune diseases could be immunized with cross-reactive antigens supposed to trigger the proliferation of autotoxic clones, and assessment of their pathogenicity studied in vivo. Moreover the importance of cells and/or cell products on the evolution of an (auto)immune response could be assessed in vivo.

#### 6. Isolating Human Antibody from hu-PBL-SCID Mice

One of the potentials of this model is to derive human monoclonal antibodies with a predetermined specificity from hu-PBL-SCID mice immunized with the desired antigen. Accordingly a number of hu-PBL-SCID mice populated with cells from donor #4, who was

immune to TT but not boosted, were immunized with TT. The mouse showing the highest IgG anti-TT level two weeks after the last boost (37 days after PBL transfer) was sacrificed.

5           To prepare a combinatorial library, RNA was first prepared from spleen, liver, kidney and peripheral blood. Reverse transcription and PCR amplification of human gamma 1 heavy chain (Fd region) and kappa light chain (Persson et al., Proc. Natl. Acad. Sci., USA, 10 88:2432-2436, 1991) confirmed the presence of human Ig mRNA in all these tissues. Spleen derived PCR products were then cloned into lambda phage vectors, and an Fab combinatorial library constructed as described by Persson et al., supra and Huse et al., 15 Science, 246:1275-1281 (1989). The combinatorial library was constructed as previously reported (Persson et al., supra), but with the addition of 3 new oligonucleotide primers for the DNA amplification of the heavy gamma 1, and k chain, respectively (VH2f= 20 5'-CAG GTG CAG CTA CTC GAG TCG GG-3' (SEQ ID NO 1); VH4f= 5'-CAG GTG CAG CTG CTC GAG TCG GG-3' (SEQ ID NO 3); VK2a= 5'GAT ATT GAG CTC ACT CAG TCT CCA-3' (SEQ ID NO 2)). The competitive ELISA assay, and nucleic acid sequencing were performed as described (Persson et 25 al., supra). This library was screened using labelled TT. Two positive clones were plaque purified and phagemids excised and used to transform E.coli. These transformants produced Fabs with apparent binding affinities of  $10^8$ - $10^9$  M<sup>-1</sup> as measured in a competition 30 ELISA. The clones were sequenced at the nucleic-acid level.

          The sequences of the clones were significantly different from those we have obtained previously from a boosted human individual arguing against the notion 35 that they were contaminants from another library.



Further, one of the clones contained a characteristic heavy chain PCR priming sequence used in this experiment for the first time. Although it is formally possible that the RNA for the positive clones arose from cells stimulated in the human host rather than in the SCID mouse, the time between PBL transfer and RNA extraction, the statistics of random combinatorial libraries, and the observed anti-TT antibody response in the hu-PBL-SCID mouse make this extremely unlikely. Only very recently boosted individuals give detectable levels of antigen-binding clones using PBL as the source of RNA. This is probably because antigen-specific cells are only found in PBL in the high proportions required for successful repertoire cloning immediately following a boost (Steven et al., J. Immunol., 122:2498-2504, 1979).

Thus, a single anti-TT positive clone was not found in a library of over a million Fabs prepared from PBL of an individual with an IgG anti-TT level of 2.42 IU/ml who had not recently been boosted (Persson et al., supra). This individual had an IgG anti-TT level similar to that of donor #4 whose PBL were used in this experiment, and who had not been boosted with TT for 17 years. In another study of an individual who had also not been recently immunized, no antigen-specific clones were found in a library of greater than a million clones prepared from the PBL of a long standing HIV positive individual with high anti-gp120 and anti-gp41 antibody titers. Two TT-specific Fabs were obtained out of a library of about 370,000 clones. This is a lower frequency than from a recently boosted human (about 1 in 5,000) but a higher frequency is anticipated when conditions in the hu-PBL-SCID mice are optimized. For example RNA extracted immediately prior to the peak of IgG

production should assist in this regard.

5 This invention has clear implications for the generation of human monoclonal antibodies. Currently repertoire cloning accesses specificities where boosting of humans is practical e.g. vaccination materials and infectious agents such as not generally dangerous viruses. The use of hu-PBL-SCID mice in conjunction with repertoire cloning should allow access to specificities where antigen contact has been made previously but boosting in humans is not feasible. For example monoclonal antibodies against HIV could be generated by using an HIV positive individual as donor and boosting in mice with virus or viral proteins. Antibodies against many self antigens, such as anti-lymphocyte surface molecules, could be produced using suitable autoimmune donors. Finally, monoclonal antibodies against MHC antigens may be generated from selected multiparous women, or transplant recipients as PBL donors.

20

The foregoing specification, including the specific embodiments and examples, is intended to be illustrative of the present invention and is not to be taken as limiting. Numerous other variations and modifications can be effected without departing from the true spirit and scope of the invention.

25

What is Claimed Is:

5           1.    A method for producing human antibodies that immunoreact with a preselected antigen in a severe combined immunodeficiency (SCID) mouse comprising the steps of:

                  a)    isolating a suspension of viable human peripheral blood lymphocytes (PBL) from a human donor;  
                  b)    contacting said viable PBL in a medium in which lymphocytes are viable with said preselected  
10           antigen in an amount sufficient for said antigen to induce an immune response in said PBL;

                  c)    intraperitoneally introducing  $2 \times 10^6$  to  $50 \times 10^6$  of said PBL into a mouse having severe combined immunodeficiency, thereby forming a hu-PBL-  
15           SCID mouse having a reconstituted human immune system; and

                  d)    immunizing said hu-PBL-SCID mouse with said antigen in an amount sufficient to induce an immune response wherein human antibodies  
20           immunoreactive with said antigen are formed in said hu-PBL-SCID mouse.

          2.    The method of claim 1 that includes the further step of harvesting said human antibodies from said hu-PBL-SCID mouse.

25           3.    The method of claim 2 wherein said harvesting comprises collecting the sera from said hu-PBL-SCID mouse.

          4.    The method of claim 2 wherein said harvesting comprises isolating a repertoire of nucleic  
30           acid sequences that encode a population of different antibody molecules from the antibody producing cells of said mouse, and expressing the different nucleic acid sequences to form said human antibodies.

          5.    The method of claim 1 wherein said isolating  
35           of step (a) comprises:

i) lymphopheresing blood from a human donor to produce lymphopheresed cells containing lymphocytes, plasma and red blood cells;

5 ii) centrifuging said lymphopheresed cells through a medium in which lymphocytes are viable at a gravity force and for a time sufficient to pellet the lymphocytes but not the red blood cells present in said lymphopheresed cells and form pelleted lymphocytes and a RBC/plasma-containing supernatant;

10 iii) separating the pelleted lymphocytes from the supernatant to form isolated PBL; and

iv) resuspending said isolated PBL in a medium in which lymphocytes are viable to form said suspension of isolated viable PBL.

15 6. The method of claim 1 wherein said isolating of step (a) comprises:

i) centrifuging heparinized whole blood from said donor through a high density ficoll medium of density 1.119 gram per milliliter to form upper, lower and inter layers;

20 ii) recovering said inter layer containing lymphocytes, plasma and red blood cells;

25 ii) diluting said inter layer with an equal volume of medium in which lymphocytes are viable;

iii) centrifuging said diluted inter layer at a gravity force and for a time sufficient to pellet the lymphocytes but not the red blood cells present in said inter layer and form pelleted lymphocytes and a RBC/plasma-containing supernatant;

30 iv) separating the pelleted lymphocytes from the supernatant to form isolated lymphocytes; and

35 v) resuspending said isolated

lymphocytes in medium in which lymphocytes are viable to form said suspension of isolated viable PBL.

7. The method of claim 1 wherein said human donor is seronegative for Epstein-Barr virus.

5           8. The method of claim 1 wherein said contacting of step (b) comprises in vitro incubating said PBL with said antigen at a concentration of about 0.1 to 20 ug antigen/ml and about 1 to  $10 \times 10^6$  PBL cells/ml under biological conditions for a time period  
10 sufficient for said antigen to induce a human immune response in said PBL.

9. The method of claim 8 wherein said contacting time period of step (b) is 1 to 6 hours.

15           10. The method of claim 1 wherein said contacting of step (b) and said introducing of step (c) are conducted substantially simultaneously by co-injection intraperitoneally into said mouse.

20           11. The method of claim 1 wherein said introducing of step (c) comprises injecting  $50 \times 10^6$  PBL intraperitoneally into said mouse.

12. The method of claim 2 wherein said harvesting comprises the steps of:

- a) isolating messenger RNA from human antibody-producing cells of said hu-PBL-SCID mouse;
- 25           b) isolating from said isolated messenger RNA a repertoire of nucleic acid sequences that encode a repertoire of different human antibodies;
- c) expressing said repertoire of human antibodies from said nucleic acid sequences in the  
30 form of a population of clones, each monoclonal with respect to an antibody;
- d) selecting a clone from said population of clones for the presence of antibody that immunoreacts with said preselected antigen;
- 35           e) culturing said selected clone to

express human antibody immunoreactive with the  
preselected antigen in the culture medium; and

5 f) collecting said expressed human  
antibody from said culture medium to form said human  
antibody.

13. The method of claim 12 wherein said clone  
expressing said human antibody molecule is a bacterial  
cell.

10 14. A reconstituted hu-PBL-SCID mouse containing  
human lymphocytes, said hu-PBL-SCID mouse having a  
plasma concentration of 2 to 5 grams per liter of  
human immunoglobulin and having plasma that is  
seronegative for Epstein-Barr virus.

15 15. A method for producing a hu-PBL-SCID mouse  
having a reconstituted human immune system defined by  
a plasma concentration of human immunoglobulin of 2 to  
5 grams per liter comprising the steps of:

20 a) lymphopheresing blood from a human  
donor to produce lymphopheresed cells containing  
lymphocytes, plasma and red blood cells;

25 b) centrifuging said lymphopheresed cells  
through a medium in which lymphocytes are viable at a  
gravity force and for a time sufficient to pellet the  
lymphocytes but not the red blood cells present in  
said lymphopheresed cells and form pelleted  
lymphocytes and a RBC/plasma-containing supernatant;

c) separating the pelleted lymphocytes  
from the supernatant to form isolated PBL;

30 d) resuspending said isolated PBL in an  
medium in which lymphocytes are viable to form said  
suspension of isolated viable PBL; and

35 e) introducing  $2 \times 10^6$  to  $50 \times 10^6$  of said  
isolated viable PBL in said suspension into a mouse  
having severe combined immunodeficiency, thereby  
forming a hu-PBL-SCID mouse having a reconstituted

human immune system.

16. The method of claim 15 wherein said human donor is seronegative for Epstein-Barr virus.

5 17. A method for producing a hu-PBL-SCID mouse having a reconstituted human immune system defined by a plasma concentration of human immunoglobulin of 2 to 5 grams per liter comprising the steps of:

10 a) centrifuging heparinized whole blood from said donor through a high density ficoll medium of density 1.119 gram per milliliter to form upper, lower and inter layers;

b) recovering said inter layer containing lymphocytes, plasma and red blood cells;

15 c) diluting said inter layer with an equal volume of medium in which lymphocytes are viable;

d) centrifuging said diluted inter layer at a gravity force and for a time sufficient to pellet the lymphocytes but not the red blood cells present in said inter layer and form pelleted lymphocytes and a RBC/plasma-containing supernatant;

e) separating the pelleted lymphocytes from the supernatant to form isolated lymphocytes;

25 f) resuspending said isolated lymphocytes in medium in which lymphocytes are viable to form said suspension of isolated viable PBL; and

30 g) introducing  $10 \times 10^6$  to  $50 \times 10^6$  of said isolated viable PBL in said suspension into a mouse having severe combined immunodeficiency, thereby forming a hu-PBL-SCID mouse having a reconstituted human immune system.

18. The method of claim 17 wherein said human donor is seronegative for Epstein-Barr virus.

1 / 3

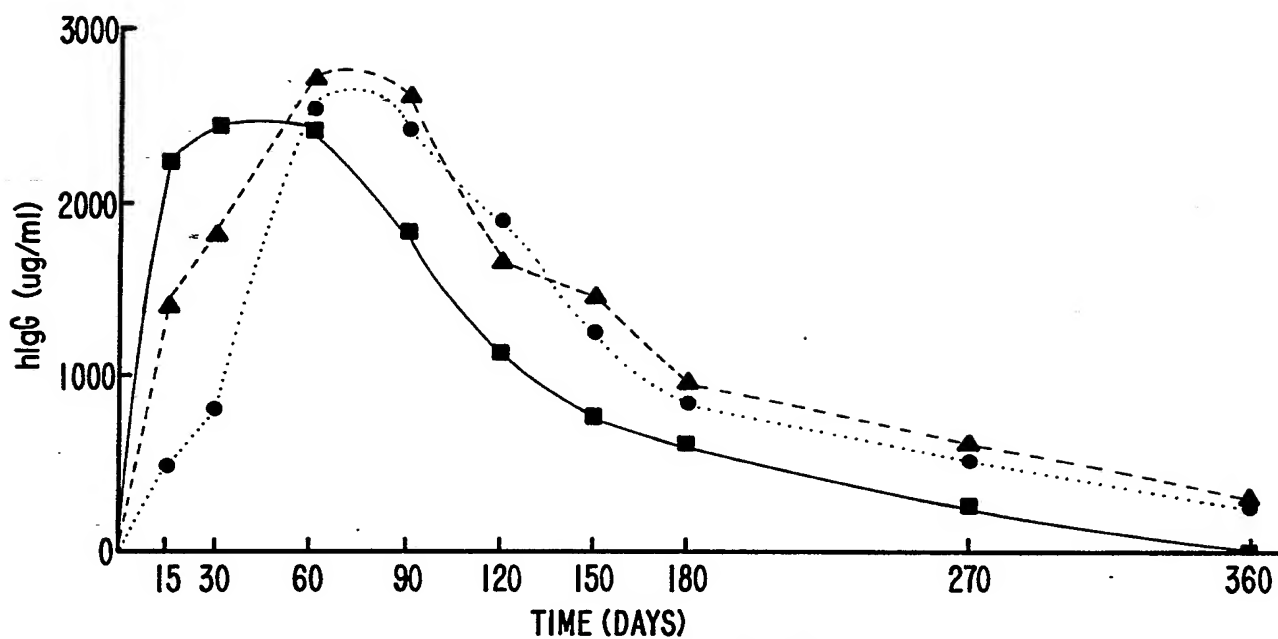


FIG. 1

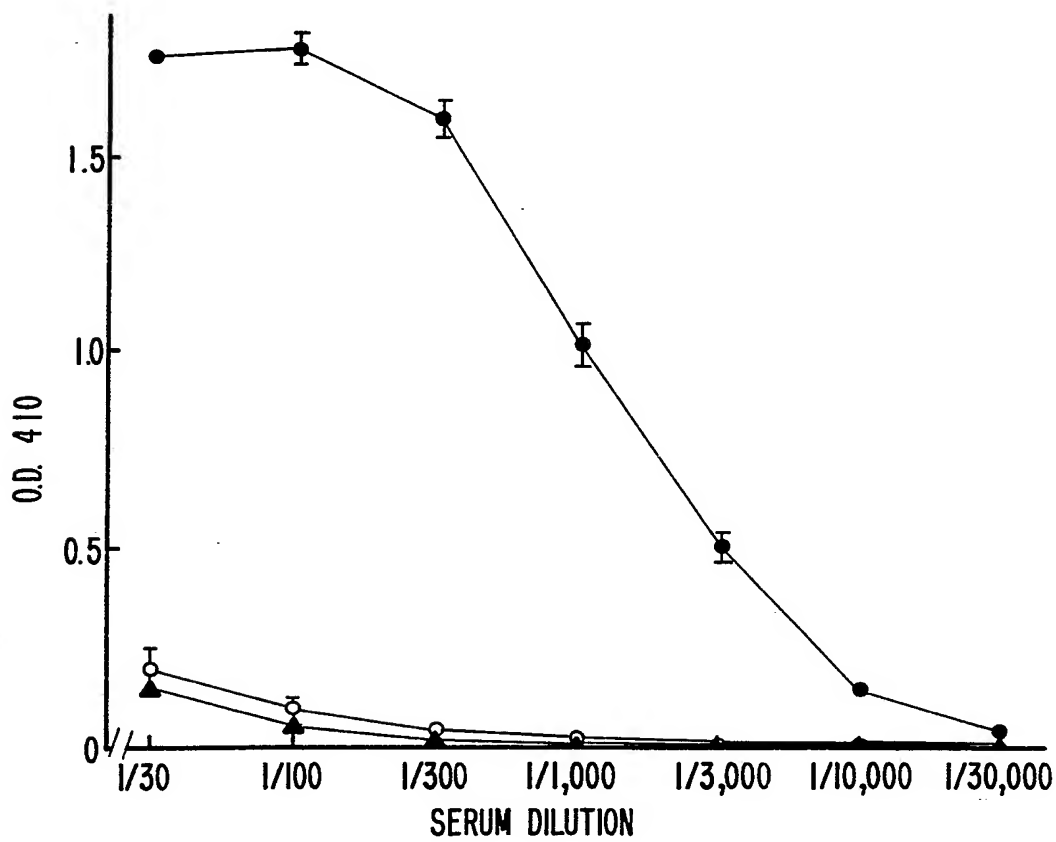


FIG. 2A

SUBSTITUTE SHEET



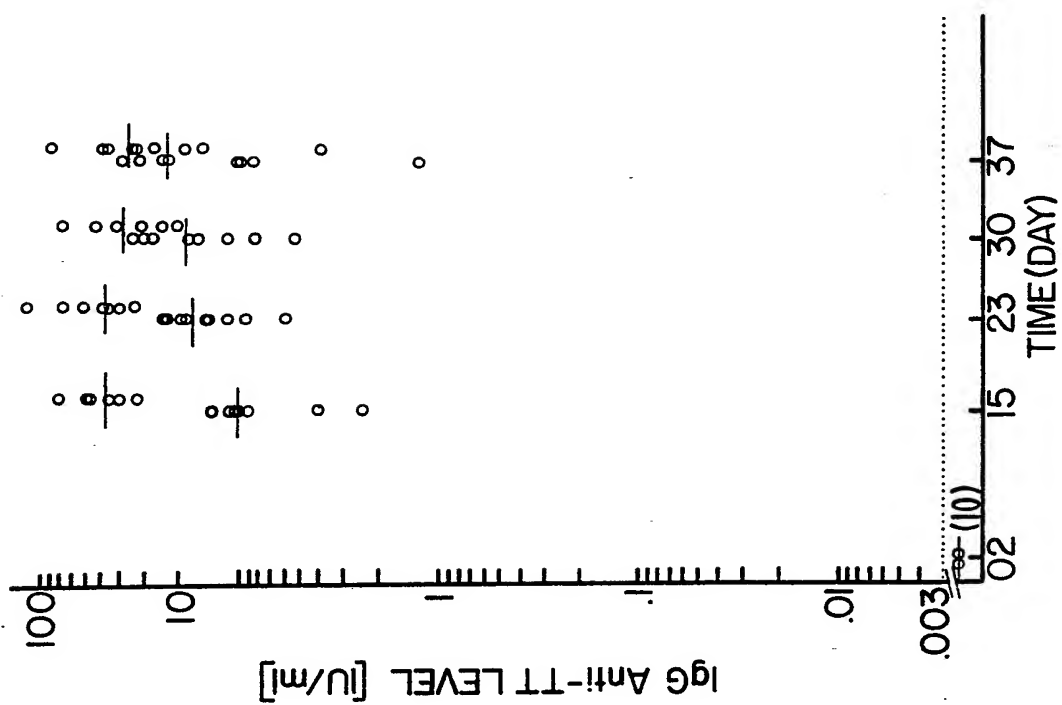


FIG. 2C

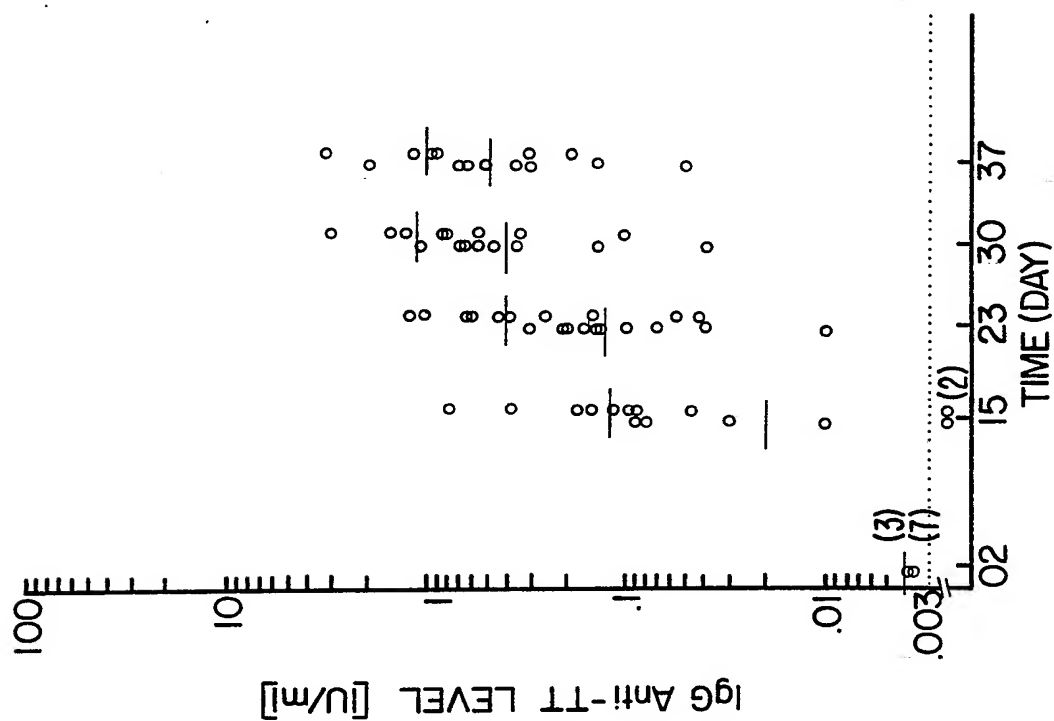


FIG. 2B

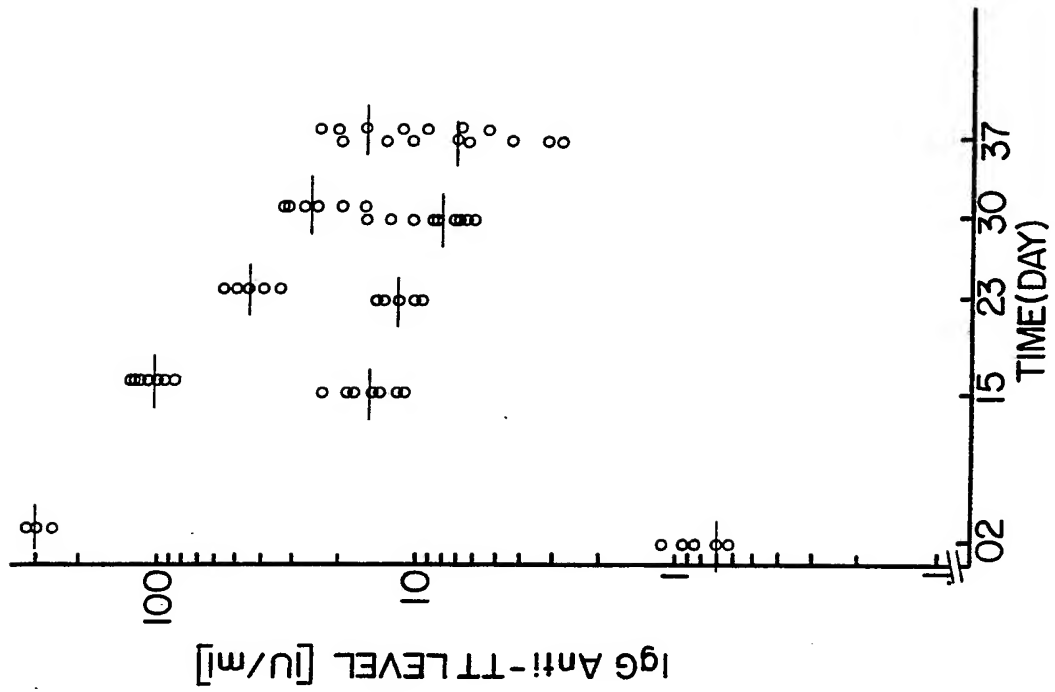


FIG. 2E

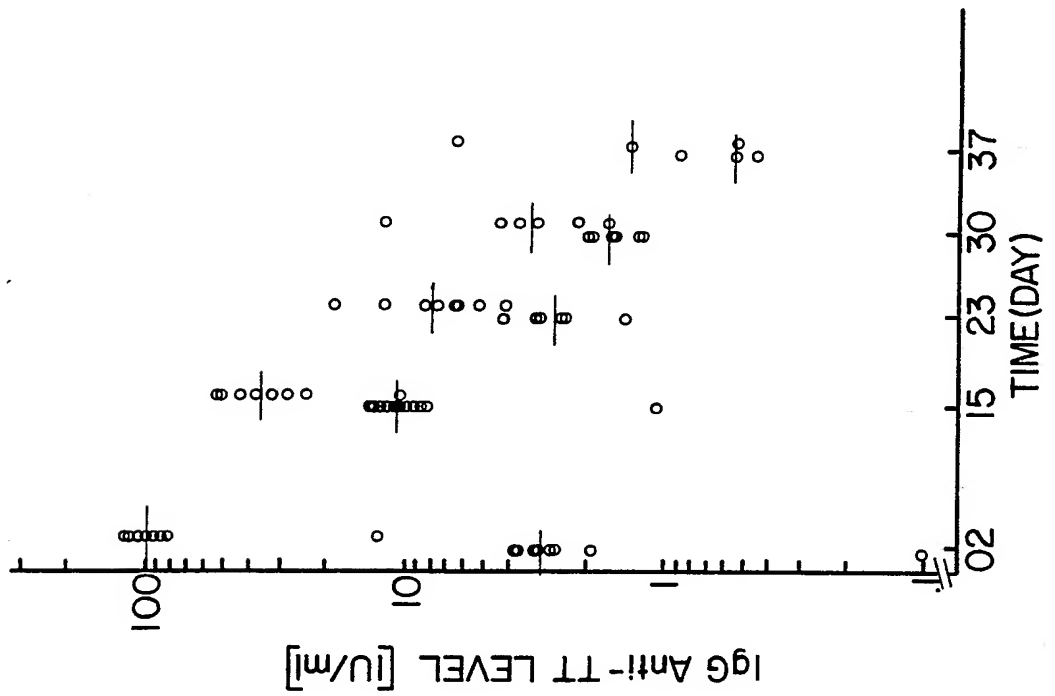


FIG. 2D

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/08005**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :A61K 35/00, A16K 39/00

US CL :800/2; 424/88, 93U

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2, DIG 5; 424/88, 93U; 435/172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 335, Issued 15 September 1988, Mosier et al., "Transfer of a Functional Human Immune System to Mice with Severe Combined Immunodeficiency", pages 256-259, see the entire document.	1 - 18
Y	Progress in Immunology, Volume 7, Issued 1989, Mosier et al., "Elements of the Human Immune System: Studies of Mature Lymphoid Cells following Xenotransplantation to SCID Mice", pages 1264-1271, see the entire document.	1 - 18
Y	J. Exp. Med., Volume 170, Issued December 1989, Krams et al., "Generation of Biliary Lesions after transfer of Human Lymphocytes into Severe Combined Immunodeficient (SCID) Mice", pages 1919-1930, see the entire document.	1 - 18
Y	Engleman et al., eds, "Human Hybridomas and Monoclonal Antibodies", published 1985 by Plenum Press(New York), pages 149-161 and 162, entire article.	1 - 18



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

26 OCTOBER 1992

Date of mailing of the international search report

02 DEC 1992

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/08005

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci. USA, Volume 87, Issued October 1990, Mullinax et al., "Identification of Human Antibody Fragment Clones Specific for Tetanus Toxoid in a Bacteriophage (Lambda) Immunoexpression Library", pages 8095-8099, see the entire document.	4, 12, 13
Y	Science, Volume 246, Issued 08 December 1989, Huse et al., "Generation of a Large Combinatorial Library of the Immunoglobulin Repertovire in Phage Lambda", pages 1275-1281, see the entire document.	4, 12, 13

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG (FILES 154, 55, 311, 312)

U.S. AUTOMATED PATENT SYSTEM (FILE USPAT, 1975 - 1992)

SEARCH TERMS: SCID, ANTIBODIES, XENOGENEIC, TRANSPLANTATION,  
LYMPHOCYTES, ANTIGEN, MOUSE, IMMUNOGLOBULIN.